The role of carnitine in eukaryotic cells: Using yeast as a model

by

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Declaration

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Summary

Previous studies in yeast in this laboratory have found carnitine to be both protective against oxidative stress induced by hydrogen peroxide and to increase the detrimental effect of dithiothreitol. These phenotypes were found to be independent of the role of carnitine within the carnitine shuttle. A screen for suppressor mutations for these carnitine-dependent phenotypes identified, among others, Δ*cho2* and Δ*opi3*. Cho2p and Opi3p catalyse the sequential methylation reactions in the formation of phosphatidylcholine from phosphatidylethanolamine.

Therefore, this study aimed to investigate the relationship between choline, phosphatidylcholine and the carnitine phenotypes. Liquid growth assays of Δ*cho2* and Δ*opi3* cultures revealed that addition of choline can restore the protective effects of carnitine against hydrogen peroxide. The connection between the cellular phospholipid composition and the carnitine-dependent shuttle-independent phenotypes was also investigated. Analysis of the lipid composition of cells by LCMS showed that Δ*cho2* and Δ*opi3* had a largely different lipid composition compared with the wild type, most notably, a reduction in phosphatidylcholine and an increase in triacylglycerol content were observed for both mutants. These changes were reversed by supplementation with choline. However, no effects on the lipid composition of cells in response to carnitine treatment were observed, either when supplemented alone or in combination with DTT and hydrogen peroxide.

Carnitine has also been investigated in mammalian systems for its potential to protect cells from oxidative stress, an effect which would be of benefit in various neurodegenerative disorders. Several studies have documented the positive effects of carnitine against oxidative stress in mammalian cells however the mechanism behind this action remains unknown. It is therefore thought that, provided similar effects for carnitine can be shown in mammalian cells as was observed in yeast, it would be beneficial to use yeast as a model system for the study of the molecular changes induced by carnitine. In view of this, the effects of carnitine on toxicity induced by oxidative stress in mammalian neural cells were compared to that which has been observed in yeast. For this purpose the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, a measure of reductive capacity of cells, was used. However, no effects for carnitine were observed in the MTT assay in combination with either dithiothreitol or paraquat.

Opsomming

Vorige studies op gis in hierdie laboratorium het bevind dat karnitien beskermend is teenoor oksidatiewe stres wat deur waterstofperoksied geïnduseer word en ook die nadelige effek van ditiotreitol verhoog. Hierdie fenotipes is gevind om onafhanklik te wees van die rol van karnitien binne die karnitien-pendel. Die sifting vir onderdrukker-mutasies van hierdie karnitienafhanklike fenotipes het onder andere Δ*cho2* en Δ*opi3* geïdentifiseer. Cho2p en Opi3p kataliseer die opvolgende metileringsreaksies tydens die vorming van fosfatidielcholien vanaf fosfatidieletanolamien.

Hierdie studie het dus gepoog om die verhouding tussen cholien, fosfatidielcholien en die karnitienfenotipes te ondersoek. Vloeistofanalises van Δ*cho2*- en Δ*opi3*-kulture het aangedui dat die byvoeging van cholien die beskermende effekte van karnitien teenoor waterstofperoksied kan herstel. Die verband tussen die sellulêre fosfolipiedsamestelling en die karnitienafhanklike pendel-onafhanklike fenotipes is ook ondersoek. Die analise van die lipiedsamestelling van selle deur middel van LCMS het getoon dat Δ*cho2* en Δ*opi3* ‘n grootliks verskillende samestelling het in vergelyking met die wilde tipe, en daar is veral ‘n afname in fosfatidielcholien en ‘n verhoging in triasielgliserol-inhoud vir beide mutante waargeneem. Hierdie veranderinge is omgekeer deur aanvulling met cholien. Geen effekte op die lipiedsamestelling van die selle is egter in reaksie op die karnitienbehandelings waargeneem nie, hetsy toe dit alleen aangevul is of in kombinasie met ditiotreitol en waterstofperoksied.

Karnitien is ook in soogdierstelsels ondersoek vir sy potensiaal om selle teen oksidatiewe stres te beskerm, ‘n effek wat groot voordeel sal inhou vir verskeie neurodegeneratiewe steurings. Verskeie studies het reeds die positiewe effekte van karnitien teen oksidatiewe stres in soogdierselle opgeteken, hoewel die meganisme agter hierdie werking nog onbekend is. Daar word dus vermoed dat, gegewe dat soortgelyke effekte vir karnitien in soogdierselle getoon kan word as wat in gis waargeneem is, dit voordelig sou wees om gis as ‘n modelsisteem vir die studie van die molekulêre veranderinge wat deur karnitien geïnduseer word, te gebruik. In die lig hiervan is die effekte van karnitien op giftigheid wat deur oksidatiewe stres in soogdiersenuselle geïnduseer is, vergelyk met dít wat in gis waargeneem is. Om hierdie rede is die 3-[4,5-dimetieltiasool-2-iel]-2,5-difeniel tetrasoliumbromied (MTT) essaiëring, ‘n meting van die verminderende kapasiteit van selle, gebruik. Geen effekte vir karnitien is egter met die MTT essaiëring in kombinasie met óf ditiotreitol óf parakwat waargeneem nie.

This thesis is dedicated to
Paul and Janette du Plessis

Biographical sketch

Michelle du Plessis was born in Middelburg, South Africa on 12 July 1991. She matriculated from Liberty Christian College in 2009 and then went on to complete a BSc degree in Molecular Biology and Biotechnology at the University of Stellenbosch in 2012. In 2013 she enrolled for a HonsBSc-degree at the Institute for Wine Biotechnology, Stellenbosch University.

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Preface

This thesis is presented as a compilation of 5 chapters.

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Investigating the effects of carnitine during oxidative stress in *Saccharomyces cerevisiae*: Membrane lipid composition and the relationship with choline

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CHAPTER 1: GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Introduction

Oxidative stress and its impact on cellular functioning have been widely studied due to its role during aging and neurodegeneration (Jones *et al.* 2010; Virmani and Binienda 2004). Many compounds have been investigated for their potential to alleviate oxidative stress, including L-carnitine (Sachan *et al.* 2012). Carnitine is most well-known for its role in the carnitine shuttle where it is bound to activated fatty acyl residues as a means of transporting them across cell membranes (Vaz and Wanders 2002). It has also been found, both in yeast and mammalian systems, to have a protective effect against oxidative stress (Franken *et al*. 2008; Li *et al.* 2012). Due to the observed protective effects of carnitine against oxidative stress, it has been a compound of interest in studies investigating neurodegeneration, as these diseases are usually associated with oxidative stress in the brain (Pettegrew *et al.* 2000; Schöls *et al*. 2004). However, the underlying mechanism behind this effect remains unclear.

In yeast, studies from this laboratory have described both protective and detrimental effects for carnitine supplementation, and these effects were found to be independent of the carnitine shuttle (Franken and Bauer 2010). When carnitine is added to media containing hydrogen peroxide, a protective effect is observed. However, supplementation of carnitine to medium containing dithiothreitol (DTT) increases the detrimental effects of this compound on yeast. Genetic screening revealed that certain mutants, among which are Δ*opi3* and Δ*cho2*, suppress the carnitine phenotypes. Opi3p and Cho2p catalyse the sequential methylation of phosphatidylethanolamine to produce the abundant membrane phospholipid phosphatidylcholine (PC) (Henry *et al.* 2012). The mutants Δ*opi3* and Δ*cho2* therefore lack PC unless provided with external choline which can be taken up and used to produce PC via the Kennedy pathway.

Saccharomyces cerevisiae is well established as a genetic model system to study fundamental cellular mechanisms, and has already been suggested as a model for the study of neurodegeneration (Bharadwaj *et al*. 2010; D'Angelo *et al.* 2013). The study of oxidative stress and other disease related effects within yeast is facilitated through the ease of working with this organism and the availability of an unmatched range of molecular tools (Tenreiro and Outeiro 2010). This laboratory has in the past studied the role of carnitine in yeast, including the cloning of the genes encoding the enzymes involved in the carnitine shuttle (Swiegers *et al.* 2002), and has more recently characterised the effect of carnitine during oxidative stress (Franken *et al*. 2008; Franken and Bauer 2010). However, the effect of carnitine during oxidative stress in yeast and mammalian cells has not, to date, been directly compared and it is unknown whether the effects observed for carnitine supplementation in the yeast and mammalian systems are the result of a common mechanism of action.

1.2 Aims of this study

The current study further investigates the link between carnitine, the *OPI3* and *CHO2* genes and PC biosynthesis while also comparing the effects of carnitine on oxidative stress in mammalian and yeast cells. More specifically, the aims were to:

- Evaluate the link between carnitine and phosphatidylcholine biosynthesis in the presence and absence of oxidative stress, and to investigate the relation of choline and PC with the carnitine shuttle-independent phenotypes
- Analyse the relationship between the phospholipid composition of cells and carnitine supplementation and carnitine-dependent phenotypes
- Compare the effect of carnitine on toxicity induced by oxidative stress in mammalian cell cultures with those observed in yeast

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CHAPTER 2: LITERATURE REVIEW

Lipid metabolism of *S.cerevisiae* and effect of environmental stress factors on cell membrane lipid composition

2.1 Introduction

The cell membrane is vital to the survival and functioning of yeast cells as it forms a barrier between the intracellular constituents of the cell and its environment. As such, membrane composition and structure are central in determining the resistance of a cell to external stresses (Chatterjee *et al.* 2000). The physical properties of membranes affect the membrane barrier function, the activity of membrane-associated enzymes as well as membrane fission and fusion (De Kroon 2007). As a result of these crucial roles, the structure and composition are governed by numerous regulatory mechanisms which play an important role in the resistance of cells to environmental stress factors (Beales 2004; Chatterjee *et al*. 2000; Lindberg *et al*. 2013).

The cell membrane is largely composed of sterols, proteins and phospholipids (Henderson *et al.* 2011). Sterols contribute to the rigidity of the membrane and in yeast this group is composed mainly of ergosterol (Van der Rest *et al.* 1995). There are also many proteins associated with the cell membrane. These are important for transport of compounds into and out of the cell, cell signalling, anchoring of the cytoskeleton and cell wall synthesis and repair (Van der Rest *et al.* 1995).

It is said that 70% of the cell membrane's lipid component is composed of phospholipids, which contribute significantly to membrane structure, and affect many important processes including signalling pathways and the transport of compounds into and out of the cell (Henderson *et al.* 2011; de Kroon *et al.* 2013). The lipid component of the yeast cell membrane is composed of glycerophospholipids, sphingolipids and ergosterol (Lindberg *et al.* 2013). Each of these plays an important role within the membranes of the cell, however for the purposes of this review the focus will be on phospholipids. The major phospholipids are phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) (Henry *et al*. 2012) (Figure 2.1). These major groups, in combination with a variety of fatty acyl chains, produce the assortment of phospholipids present in the cell, each with a specific function (De Kroon *et al.* 2013).

A variety of mechanisms are employed in the regulation of phospholipid synthesis, including genetic regulation, transcript abundance and phosphorylation of biosynthetic enzymes (Carman and Han 2009). In addition to these regulatory pathways, the change in phospholipid composition observed in some instances of environmental stress are thought to include sensors and signal transduction pathways, many of which have not yet been identified (Balogh *et al.* 2013; de Kroon *et al*. 2013).

This review aims to show the importance of understanding the processes governing lipid homeostasis in the cell. Lipid biosynthesis as well as the structure and roles of the major phospholipids are briefly discussed. The mechanisms employed by yeast to maintain homeostasis, as well as the manner in which phospholipid and fatty acid metabolism are altered in response to stress conditions are also discussed. Lastly, the potential for the use of *Saccharomyces cerevisiae* as a model for the study of lipid biosynthesis and regulation is considered.

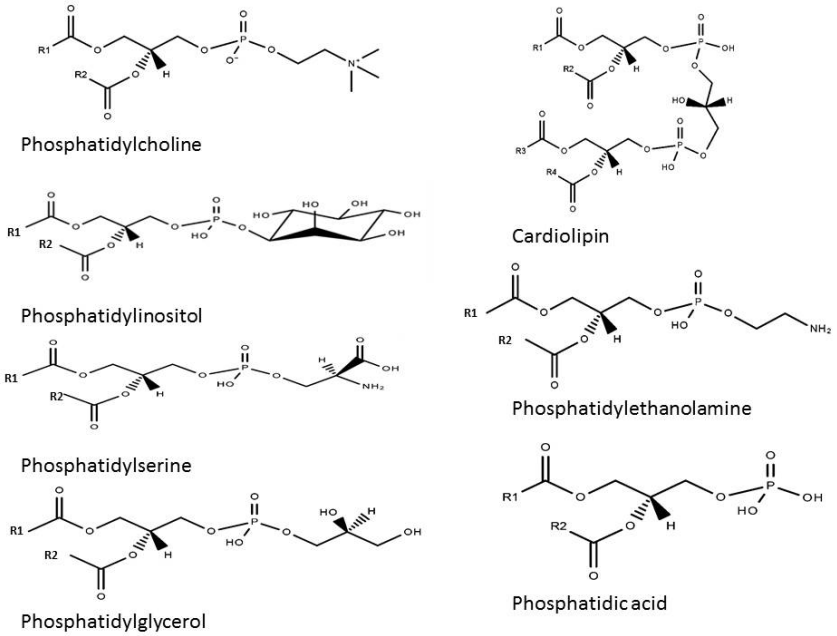

Figure 2.1: Major phospholipids: phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL), with R1, R2, R3 and R4 representing the acyl chains. Structures from Lipid Maps: (http://www.lipidmaps.org).

2.2 Phospholipid metabolism

The main phospholipids found in the cell are phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) (Henry *et al.* 2012). While PA, PE, PC and PI are distributed largely throughout all of the cellular membranes, PG is found only in the mitochondrial membranes and CL in the mitochondria and peroxisomes (Carman and Kersting 2004; Zinser *et al*. 1991). Phospholipids consist of fatty acids esterified to a glycerol-3-phosphate backbone at positions 1 and 2 (Carman and Kersting 2004). The most common fatty acids used in the synthesis of phospholipids include palmitic acid (C16-0), palmitoleic acid (C16-1), stearic acid (C18-0) and oleic acid (C18-1) (Carman and Han 2011). This section outlines fatty acid synthesis which is then followed by a brief description of phospholipid synthesis.

2.2.1 Fatty acid metabolism

In the initial step of fatty acid synthesis, malonyl-CoA is produced by condensation of acetyl-CoA with a bicarbonate anion through the action of the acetyl-CoA carboxylase (Acc1p) (Hofbauer *et al.* 2014). Malonyl-CoA is then combined with acetyl-CoA to form acyl-CoA, which may undergo elongation via the successive addition of malonyl-CoA (Beopoulos *et al.* 2011). This process is catalysed by the fatty acid synthase (FAS) enzymatic complex and takes place mainly in the cytosol, though minor amounts also occur in the mitochondria (Henry *et al.* 2012). Thereafter, desaturation takes place in the ER and is catalysed by Ole1p, a Δ9-desaturase (Kandasamy *et al*. 2004).

The major fatty acid chains produced *de novo* are 16 or 18 carbons long, although minor amounts of C_{12}, C_{14} and up to C_{26} also exist, and are either saturated or monounsaturated (Henry *et al.* 2012). Fatty acids may also be acquired through the uptake of external fatty acids or derived from the products of lipid turnover (De Kroon *et al.* 2013). These fatty acids are then used as precursors for phospholipids and TAGs (Henry *et al.* 2012).

2.2.2 Overview of phospholipid metabolism

The ER has been described as the main site for phospholipid synthesis, however, other sites of lipid synthesis also exist, including the Golgi and mitochondria (Henry *et al.* 2012). A brief overview of phospholipid metabolism will be given here. For a more thorough review on the topic, the paper by Henry and co-workers is recommended (Henry *et al.* 2012) (Figure 2.2). PA, produced from glycerol-3-phosphate, is central to phospholipid metabolism as it is the precursor in the *de novo* synthesis of all other phospholipids. PA is used in the formation of cytidine diphosphate-diacylglycerol (CDP-DAG) and diacylglyerol (DAG), which in turn are used in the synthesis of PE and PC via the CDP-DAG and Kennedy pathways respectively. In the CDP-DAG pathway, CDP-DAG is used to produce PS which undergoes decarboxylation to form PE. PE then undergoes three methylation reactions to form PC. The Kennedy pathway requires the uptake of exogenous choline and ethanolamine which then undergo phosphorylation, followed by activation by CTP and are finally combined with DAG to produce PC and PE, respectively. Moreover, CDP-DAG is also used to donate a phosphatidyl moiety to inositol to form PI, or to glycerol-3-phosphate to form phosphatidylglycerophosphate which can be converted to PG. PG can then also combine with CDP-DAG to form CL.

In addition to these biosynthetic pathways, most phospholipids also undergo rapid turnover which is accomplished through the action of phospholipases and lipid phosphatases (Carman and Han 2009). These enzymes break down phospholipids and thereby provide the cell with raw materials for the synthesis of the required phospholipids (Henry *et al*. 2012).

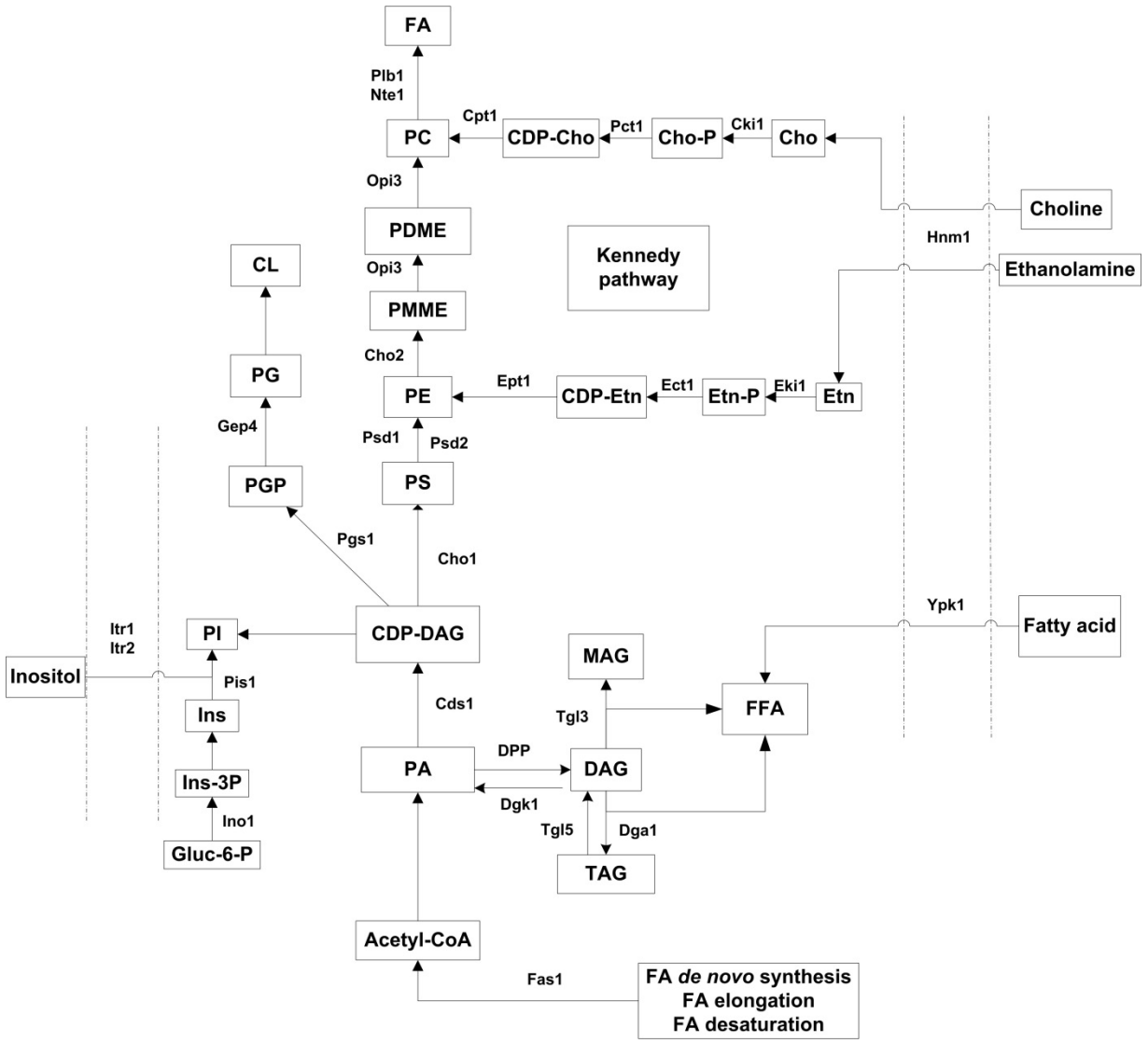

Figure 2.2: Overview of phospholipid metabolism in yeast. Abbreviations: TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerol; FFA, free fatty acids; Gluc-6-P, glucose-6-phosphate; Ins-3P, inositol-3-phosphate; Ins, inositol; PI, phosphatidylinositol; CDP-DAG, CDP-diacylglycerol; PGP, phosphatidylglycerol phosphate; PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine; PE, phosphatidylethanolamine; CDP-Etn, CDP-ethanolamine; Etn-P, ethanolamine phosphate; Etn, ethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyl-dimethylethanolamine, PC, phosphatidylcholine; FA, fatty acids; CDP-Cho, CDP-choline; Cho-P, choline phosphate; Cho, choline. Adapted from (Henry *et al.* 2012).

2.2.3 Remodelling of phospholipid acyl chain length and saturation

Within each of the major phospholipid classes, a variety of molecular species with fatty acyl chains of different lengths and levels of desaturation exist (German *et al.* 2007). This complex mixture of molecular species within each of the phospholipid classes is not merely the result of the different biosynthetic routes existing for each phospholipid, but significant evidence points to post synthetic remodelling of the acyl chains to produce the required profile for each phospholipid class (Boumann *et al*. 2003; de Kroon *et al*. 2013). This remodelling and turnover of phospholipids to

produce a specific species profile is accomplished through the action of phospholipases, acyltransferases, and transacylases (Henry *et al*. 2012).

Acc1p, which catalyses the first and rate-limiting step in fatty acid synthesis, has been found to regulate the ratio of C16:C18 fatty acid chains available for phospholipid synthesis (Hofbauer *et al*. 2014). It was also shown that the transcription factor Opi1p, which regulates transcription of numerous genes involved in phospholipid metabolism, preferentially binds PA with C16 fatty acyl chains. This finding indicates that the acyl chain composition of phospholipids has a broader influence on the regulation of phospholipid metabolism in general. However, despite all that has been observed thus far, the mechanisms employed in the remodelling of acyl chains are still largely unknown (De Kroon *et al.* 2013).

In addition to fatty acyl chain lengths, the level of unsaturation of the residues is also tightly regulated within the cell since unsaturated fatty acids are essential for yeast to grow (De Kroon *et al.* 2013). Monounsaturated fatty acids make up the bulk of fatty acids present in the cell (Martin *et al*. 2007). Fatty acids are desaturated through the action of the Δ9-fatty acid desaturase Ole1p, which requires the presence of oxygen (De Kroon *et al.* 2013). In addition; carbon source, temperature, cobalt, iron chelators and presence or absence of exogenous fatty acids have all been found to impact the expression of *OLE1* (Martin *et al.* 2007).

2.2.4 Phospholipid distribution and translocation

The phospholipid classes are not homogenously distributed throughout the cell membranes, and differences are found not only between membranes of the different organelles, but also between the leaflets of a given membrane bilayer (Santos and Riezman 2012). Emphasizing the importance of specific lipid composition to organelle membrane structure and function, it has been found that mutations of PA phosphatase (*PAH1*), which leads to increased PA levels, result in expansion of the nuclear/ER membrane (Santos-Rosa *et al.* 2005).

Attempts have been made to characterise the phospholipid composition of the different organelles (Schneiter *et al.* 1999; Zinser *et al.* 1991). The lipid composition of the organelle membranes were found to be largely similar, however, some differences were present, particularly in the plasma membrane where PS was approximately 30% more abundant and PC levels decreased by a similar value compared with most intracellular membranes (Zinser *et al.* 1991). Further study found the plasma membrane to be particularly enriched in saturated PE and PS molecules (Schneiter *et al*. 1999).

The mechanisms behind how cells achieve the differential phospholipid distribution are still poorly understood (Santos and Riezman 2012). However the results of some studies have provided an indication as to some of the mechanisms (De Saint-jean *et al*. 2011; Toulmay and Prinz 2012). A

protein known to be involved in lipid transport, Sec14p has been found to be involved in the sensing and regulation of membrane PI and PC (Bankaitis *et al.* 2010). Flippases transfer phospholipids from the extracellular/luminal side to the cytoplasmic side, and the opposite is accomplished by floppases (Leventis and Grinstein 2010). Scramblases catalyse these reactions in both directions and are thought to randomize distribution, however, their precise role is as yet unclear (Leventis and Grinstein 2010).

There are several challenges to address in the analysis of phospholipid distribution. These include problems with the isolation of organelles due to the difficulty of preventing cross contamination, and many of the protocols for the isolation of individual membranes are time-consuming and therefore redistribution of lipids during these procedures can occur (Leventis and Grinstein 2010). Asymmetry between bilayers also cannot be studied using these techniques and is therefore to a large extent disregarded (Leventis and Grinstein 2010). Also, some organelles are present in quantities too small for accurate representation and therefore, either cannot be included or require alteration of carbon source and growth conditions to obtain sufficient amounts which has unknown effects on the lipid composition (Zinser *et al.* 1991).

Fluorescence microscopy has emerged as one solution to these challenges. This technique has been successfully used in the study of phosphatidylethanolamine and phosphatidylserine localisations (Luo *et al.* 2009; Yeung *et al.* 2008) However, there is still a lack of appropriate tags for most phospholipids and consequently this technique is not as yet a viable alternative to most currently used methods (Leventis and Grinstein 2010; Santos and Riezman 2012).

2.3 Major Phospholipids: structure and function

The different phospholipid classes have unique structural and electrostatic features primarily conferred by the headgroup. This difference is further modulated by the use of the different acyl chains, allowing specific lipid compositions to be associated with specific functions within the cell (De Kroon *et al.* 2013). This section will include a brief discussion of each phospholipid class, its structure and the roles it has been found or is thought to play within the cell.

2.3.1 Phosphatidic acid

Phosphatidic acid is an anionic, cone shaped phospholipid and is therefore important in membrane curvature (Wang *et al.* 2006). Although PA is found in very small quantities within the cell, it is the precursor in the synthesis of all other phospholipids (Xavier da Silveira dos Santos *et al.* 2014). As such it plays an important role in the regulation of phospholipid synthesis (Henry *et al*. 2012) which will be discussed in more detail in a later section. PA is also used in cell signalling for various cellular processes including vesicular trafficking, meiosis, secretion and sporulation (Wang *et al.* 2006).

A connection between PA and the regulation of lipid droplet size has also recently been investigated (Fei *et al.* 2011). In this study, mutants with "supersized" lipid droplets (SLDs) also contained high concentrations of PA and reduction of PA through overexpression of PA phosphatases reduced the formation of SLDs. Furthermore, PA was found to induce the fusion of artificial lipid droplets in a manner dependent on the phospholipid composition of the lipid droplets, with the presence of PE promoting the formation of SLDs, indicating a link between the two phospholipids in this process (Fei *et al.* 2011). In corroboration of these results, a role for PA in mitochondrial membrane fusion events has also been shown (Choi *et al.* 2006).

2.3.2 Phosphatidylethanolamine

Phosphatidylethanolamine is the precursor for phosphatidylcholine via the CDP-DAG pathway, but has its own role independent of this and is essential for growth (Luo *et al.* 2009). Even a reduction in the quantity of PE in the cell has been shown to be detrimental to cell growth, especially on non-fermentable carbon sources (Birner *et al.* 2001). The specialised physical properties of these molecules are required to ensure physiological outcomes that are central to membrane function, explaining their requirement. Firstly, PE has a conical shape (Klose *et al.* 2012) which causes membrane curvature. Secondly, the small headgroup of this phospholipid leads to its ability to form non-bilayer hexagonal structures under physiological conditions (Deng *et al.* 2010). In addition, PE is thought to play a role in membrane biogenesis, as this phospholipid, which is usually found in the inner leaflet of the plasma membrane, has also been found to temporarily translocate to the outer leaflet during cell division (Iwamoto *et al.* 2004). Results from other studies indicate PE may influence cell division through regulation of actin cytoskeleton organisation (Luo *et al*. 2009).

It has been found that depletion of PE in the plasma membrane impacts negatively on both membrane structure and function, specifically on the transport of amino acids (Schuiki *et al.* 2010). Decreased levels of PE has also been found to impact negatively on respiration, the assembly of mitochondrial protein complexes and led to loss of mitochondrial DNA (Birner *et al.* 2003), highlighting the important role that PE plays in the mitochondria. Other roles for PE include involvement in the autophagic process (Nebauer *et al.* 2007). It also acts as a donor of phosphoethanolamine which links proteins to glycophosphatidylinositol (Iwamoto *et al*. 2004).

2.3.3 Phosphatidylcholine

Phosphatidylcholine is the most abundant phospholipid in cellular membranes and comprises over 50% of the phospholipids present (Leventis and Grinstein 2010). Therefore, the PC molecules are thought to be a major determinant of the physical properties of cell membranes (De Kroon 2007). The cylindrical shape of the PC molecule makes it ideal for use in the formation of lipid bilayers (Van der Rest *et al.* 1995). Phosphatidylcholine is also considered to be important in cellular signalling as the source of lipid signalling molecules such as lysoPC, phosphatidate, diacylglycerol, lysophosphatidate and arachidonic acid (Kent and Carman 1999).

There are two pathways for the synthesis of phosphatidylcholine, namely the CDP-DAG pathway and the Kennedy pathway (Henry *et al.* 2012). In the former, phosphatidylcholine is produced via the methylation of phosphatidylethanolamine while the latter requires the uptake of extracellular choline (Boumann *et al.* 2003). PC is in turn also catabolised by B and D type phospholipases yielding glycerophosphocholine and free fatty acids or PA and choline, respectively (Henry *et al.* 2012). Yeast use the CDP-DAG pathway for the production of PC when grown in the absence of choline (Janssen *et al.* 2002a). The nature of the acyl chains produced have been found to differ depending on the synthesis pathway, with the CDP-DAG pathway producing mainly diunsaturated PC and the Kennedy pathway primarily producing monounsaturated species (Boumann *et al.* 2003). However, it was also found that mutants for enzymes in one or the other of the pathways still have phospholipid species distributions similar to that of the wild type (Boumann *et al.* 2003). These findings provide strong evidence for the extensive post synthetic remodelling of PC to produce a specific species profile (De Kroon 2007).

The tightly-regulated synthesis and turnover of PC is of high importance as seen by the variety of disorders where dysfunction of these processes is thought to play a role, including apoptosis, Alzheimer's disease and oncogenic transformation (Boumann *et al.* 2003). In yeast, depletion of phosphatidylcholine results in respiratory deficient cells, highlighting the importance of PC in the proper functioning of the mitochondria (Griac *et al.* 1996). The specific reason for the requirement of PC in the mitochondria is unknown, however, evidence has been found for association of PC with the FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase Gut2p, which is involved in maintaining redox balance in cells under aerobic conditions, thus providing a link between PC and respiration (Janssen *et al.* 2002b). Decreased levels of PC has also been found to lead to global shortening and increased saturation of fatty acyl chains, specifically in PE which then becomes the most abundant phospholipid. This is thought to increase its ability to form bilayers, a finding which underscores the importance of PC in membrane structure (Boumann *et al*. 2006).

2.3.4 Phosphatidylserine

Phosphatidylserine is an anionic phospholipid which is mostly found within the cytoplasmic leaflet of the plasma membrane, but is also present in the endosomal and lysosomal membranes (Leventis and Grinstein 2010). PS is found in relatively low concentrations in the cell compared to the other phospholipids, however, its unique structure makes it of importance in processes such as the recruitment and activation of enzymes and structural components of cellular membranes (Maeda *et al.* 2013; Tani and Kuge 2012). These functions are mainly attributable to the electrostatic potential conferred to membranes due to its negative charge (Leventis and Grinstein 2010). Accordingly, studies indicate that PS is involved in the regulation of membrane surface charge and protein localisation (Yeung *et al.* 2008). It has also been suggested that PS may be involved, together with sphingolipids, in vesicular trafficking processes due to the irregular

distribution of the v-SNARE protein observed when synthesis of these lipids is inhibited (Tani and Kuge 2014).

2.3.5 Phosphatidylinositiol

Phosphatidylinositol is an anionic, bilayer-forming phospholipid (De Kroon *et al.* 2013). PI is an essential phospholipid and plays an important role in the regulation of numerous cellular processes (Gardocki *et al.* 2005). This is in great part due to the inositol ring which can be reversibly phosphorylated to form various phosphatidylinositolphosphates or donated for the synthesis of sphingolipids (Odorizzi *et al.* 2000). Collectively referred to as phosphoinositides, these molecules are important in various signalling and recognition processes (Odorizzi *et al.* 2000), including the glycolipid anchoring of proteins, mRNA export from the nucleus and vesicle trafficking (Gardocki *et al*. 2005) (Figure 2.3). The reversible phosphorylation of PI is also used in organelle recognition pathways and in other regulatory pathways (Van Meer *et al*. 2008).

In humans, alterations of phosphatidylinositol metabolism have been discovered in various diseases including; bipolar disorder, Scott syndrome and myelogenous leukemia (Gardocki *et al.* 2005), findings which show the importance of proper regulation of cellular PI levels. It is thought that PI synthase (*PIS1*) is regulated at the transcriptional level in response to a variety of environmental stimuli including carbon source (Anderson and Lopes 1996) and oxygen availability (Gardocki and Lopes 2003) and appears to be coordinated with peroxisome biogenesis (Gardocki *et al*. 2005).

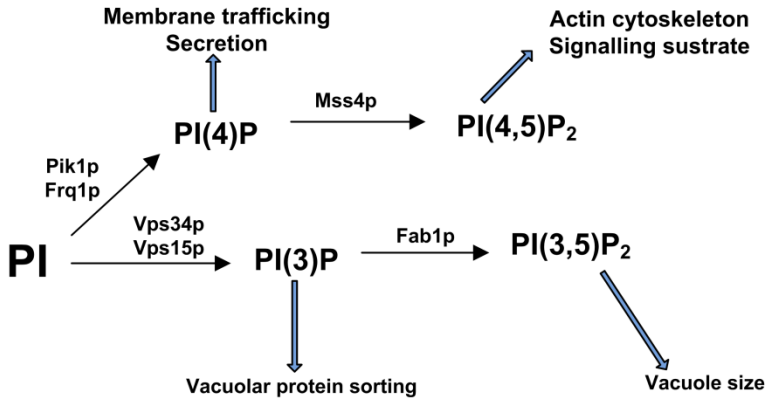

Figure 2.3: Synthesis and roles of phosphoinositides in the cell. Abbreviations: PI(4)P, phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PI(3)P, phosphatidylinositol 3-phosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate. Adapted from (Gardocki *et al.* 2005).

2.3.6 Cardiolipin and Phosphatidylglycerol

CL is an anionic phospholipid which consists of two phospholipid head groups linked to four acyl chains (Joshi *et al.* 2012). It is mainly found within the mitochondrial membranes (Henry *et al.* 2012). CL plays an important role in a variety of processes including in anaerobic metabolism and

also in mitochondrial biogenesis and function (Šimočková *et al.* 2008). In addition, CL has a shared function with PE in the fusion of mitochondrial membranes (Joshi *et al*. 2012).

PG is an anionic phospholipid which is the precursor to CL and is present in very small quantities within the cell (Šimočková *et al.* 2008). Both PG and CL are important in mitochondrial membrane permeability, solute transport and oxidative phosphorylation (Su and Dowhan 2006). Some studies indicate that PG may be able to partially compensate for loss of CL (Khalifat *et al.* 2014). However, mutants deficient in CL still exhibit decreases in mitochondrial membrane potential, respiratory rate and in ATPase and cytochrome c activities, indicating that PG cannot fully replace CL within the cell (Khalifat *et al*. 2014). Strains deficient in both PG and CL are dependent on the availability of a fermentable carbon source and are also more sensitive to changes in temperature (Su and Dowhan 2006).

2.4 Maintaining homeostasis

S. cerevisiae is described as being very tolerant to changes in its cellular membrane composition with only PI, PE and some form of methylated PE (PC or PDME) being essential (Boumann *et al*. 2006; de Kroon 2007). Various factors can impact the composition of the cellular membrane such as the growth phase of the organism as well as environmental conditions including the temperature and nutrient composition of the medium in which the yeast is growing (Klose *et al*. 2012). A high level of regulation is required in the synthesis of phospholipids due to the large number of different species which are produced from a relatively small number of precursors (Jewett *et al.* 2013). Although various mechanisms and molecules involved in regulation are described below, exactly how cells maintain the correct phospholipid composition remains largely unknown (De Kroon *et al.* 2013).

2.4.1 Transcriptional regulation

2.4.1.1 Regulation through UAS_{INO}

Numerous genes within the phospholipid synthesis pathways (*OPI3*, *CHO2*, *INO1* etc.) contain an inositol-sensitive upstream activating element (UAS_{INO}) within their promoter regions (Henry *et al.* 2012). Transcription of these genes requires binding of this region by the transcriptional activators Ino2p-Ino4p. In addition, binding of the transcription factor Opi1p to Ino2p inhibits transcriptional activation of genes containing this element. The binding of Opi1p is dependent on the translocation of this protein into the nucleus which, in turn, is regulated by the abundance of PA (Carman and Henry 2007). When the levels of PA are high, Opi1p is bound by PA and confined to the ER membrane, allowing for transcription of the UAS_{INO} containing genes. When the levels of PA are decreased, the amount of free Opi1p entering the nucleus increases, leading to a decrease in

transcription of the *UAS$_{INO}$* genes. This method of regulation is involved in many of the cellular responses for the maintenance of lipid homeostasis and is summarized below (Figure 2.4).

Although this regulatory circuit relies to a great extent on the cellular levels of PA, the enzyme phospholipase B, involved in degradation of PC, has also been shown to increase binding of PA under conditions of high PC synthesis independently of the levels of PA (Fernandez-Murray *et al*. 2009). The mechanism behind this is unknown though it has been suggested that this could be due to the resultant change of phospholipid composition in the ER membrane (Fernandez-Murray *et al.* 2009) or from the increased negative charge of the PA present which occurs due to the changes in membrane composition (De Kroon *et al*. 2013).

2.4.1.2 Regulation by zinc

Zinc plays a role in phospholipid metabolism, not only as a cofactor for metabolic enzymes but also as a structural component of various proteins including lipid binding proteins and transcription factors (Henry *et al.* 2012). The regulation of the PI synthase (*PIS1*) gene by zinc is achieved through a zinc-sensing transcriptional activator Zap1p and a zinc-responsive cis-acting element (*UAS$_{zre}$*) (Figure 2.4c). Depletion of zinc leads to an increased synthesis of PI which depletes PA and therefore leads to decreased transcription of *UAS$_{INO}$*-containing genes, this leads to a decreased synthesis of PE (Iwanyshyn *et al*. 2004). However, PC is not much affected due to induction of the Kennedy pathway (Henry *et al.* 2012). Transcription of PA phosphatase (*PAH1*) has also been found to be upregulated by depletion of zinc, a response which further represses *UAS$_{INO}$* genes (Soto-Cardalda *et al.* 2011).

2.4.1.3 Water soluble precursors of glycerophospholipid metabolism.

The synthesis of phospholipids is also governed by the availability of a number of water soluble precursors of phospholipid metabolism including inositol, choline, CTP and s-adenocyl methionine (ado-met) (Carman and Han 2009). These precursors regulate lipid metabolism in various ways, including at the level transcription, enzyme activity and metabolic flux (Henry *et al*. 2012).

Choline and inositol appear to have interrelated roles in the regulation of phospholipid synthesis, and supplementation of these precursors alone and in combination have been previously studied (Gardocki *et al*. 2005). Inositol supplementation leads to repression of the inositol-3-P synthase (*INO1*) and an increase in the synthesis of PI through induction of PI synthase (Santiago and Mamoun 2003). The increased synthesis of PI leads to a depletion of PA which allows Opi1p to enter the nucleus resulting in a further increase in PI production and a decrease in PA, PS and PC (Figure 4c). Supplementation of choline alone affects the rate and mechanism of PC turnover (Dowd *et al.* 2001) but has little effect on the phospholipid synthesis genes (Greenberg and Lopes 1996). Choline, in addition to inositol leads to an amplified response to that of inositol alone and further increases the repression of *INO1* and the *UAS$_{INO}$* genes (Santiago and Mamoun 2003).

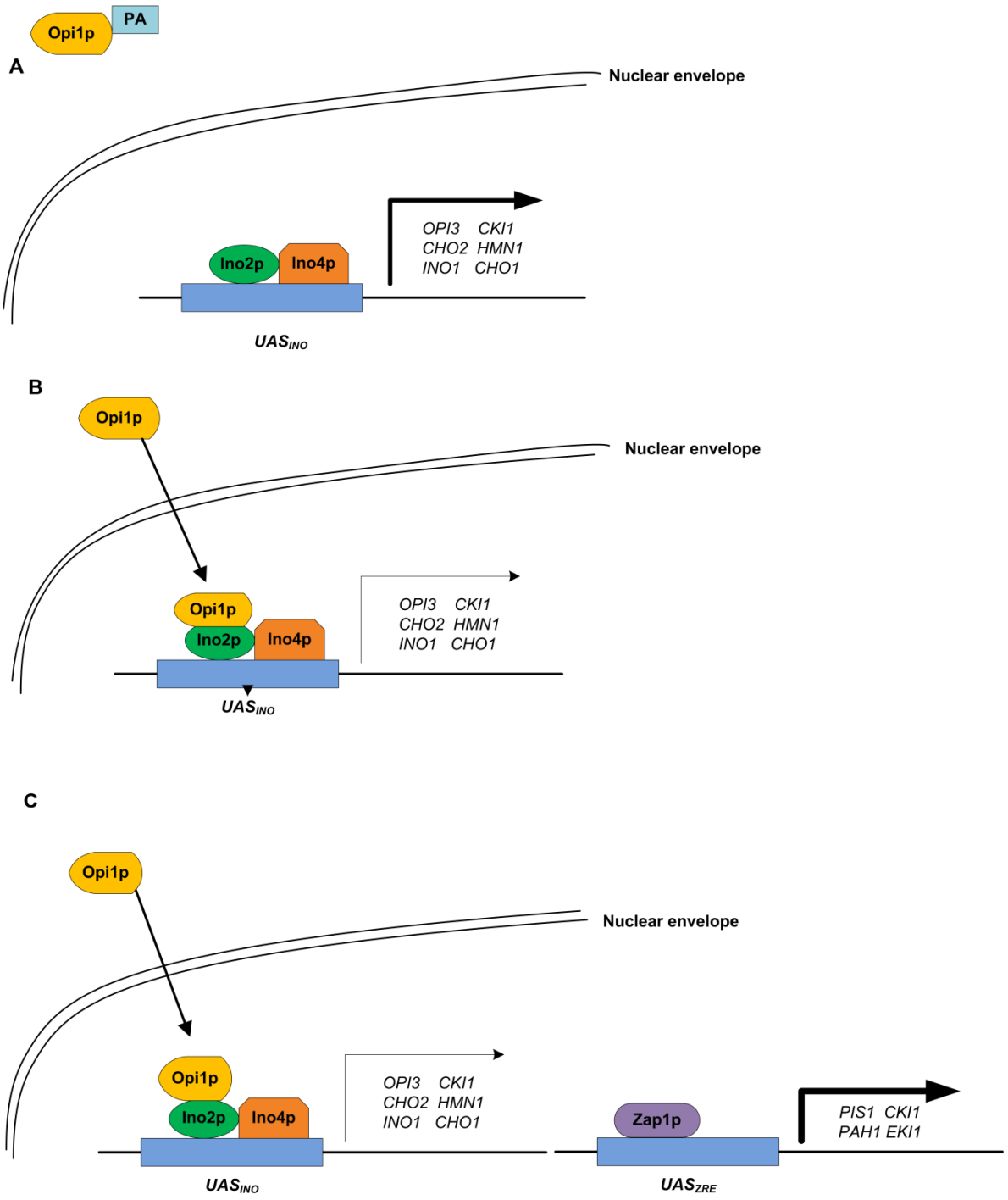

Figure 2.4: Regulation of *UAS*$_{INO}$ genes. A: PA binds to the Opi1p, preventing its entry into the nucleus, resulting in increased transcription of *UAS*$_{INO}$ genes. B: when PA levels are low, Opi1p enters the nucleus, binding the transcriptional activator Ino2p resulting in inhibition of transcription. C: when zinc levels in the cell are depleted, activation of the *UAS*$_{ZRE}$ by Zap1p results in increased synthesis of PI, depleting PA and allowing Opi1p to repress transcription of *UAS*$_{INO}$ genes. Adapted from (Carman & Han 2011).

2.4.2 Post transcriptional regulation

2.4.2.1 Transcript abundance though mRNA degradation

The genes associated with lipid biosynthesis which have been found to display this type of regulation include *CHO1* and *OLE1* (Henry *et al.* 2012). The stability of the *CHO1* mRNA was shown to increase significantly in cells containing mutations in respiration, resulting in increased levels of PS synthase (Choi and Carman 2007), an indication that this enzyme is regulated by the respiratory state of the cell. The *OLE1* mRNA displays decreased stability when unsaturated fatty acids are supplied exogenously (Kandasamy *et al.* 2004). Whereas the deletion of fatty acid synthase genes (*FAA*1 and *FAA*4) leads to increases in the levels of *OLE1* mRNA, regardless of the presence of supplementation with unsaturated fatty acids (Faergeman *et al*. 2001).

2.4.3 Enzymatic regulation

2.4.3.1 Phosphorylation of biosynthetic enzymes

Lipid biosynthesis is also controlled through the phosphorylation state of numerous biosynthetic enzymes as well as some proteins involved in the transcription of related genes (Carman and Han 2011). Most of the phosphorylation activity is carried out through the action of protein kinase A or C (Carman and Kersting 2004), but casein kinase II and a cyclin-dependant kinase have also been showed to be involved (De Kroon *et al.* 2013). A number of the enzymes which are either activated or inhibited through phosphorylation are summarized in Table 2.1. Phosphorylation of PA phosphatase (Pah1p) is also the mechanism by which the cellular localisation of this enzyme is controlled: the phosphorylated form being cytosolic and the dephosphorylated form associated with the membrane (Choi *et al.* 2011). The enzyme responsible for regulation of fatty acid chain length, *ACC1*, is also tightly regulated through the action of the kinase Snf1p in response to both salt stress and glucose limitation (Hofbauer *et al.* 2014).

2.4.3.2 Water soluble precursors

CTP is essential for phospholipid synthesis as a precursor for CDP-DAG, CDP-choline/ethanolamine and as a phosphate donor for the synthesis of PA (Henry *et al*. 2012). CTP also acts as an inhibitor of PAH1-encoded PA phosphatase activity (Kent and Carman 1999). Increases in CTP levels leads to higher levels of PA which results in derepression of UAS_{INO} genes. CTP also plays a specific and important regulatory role in PC synthesis as the substrate for the rate limiting step of PC synthesis via the Kennedy pathway and as the inhibitor of serine synthase (Cho1p).

Another regulator of PC synthesis is *S*-andenosyl-$_L$-homocysteine (ado-hcy) (Carman and Han 2009). Ado-hcy is formed from adenosyl-methionine via the methylation reactions catalysed by the methyltransferase enzymes; Cho2p and Opi3p in the CDP-DAG pathway (Carman and Han 2011).

Ado-hcy is a competitive inhibitor of these enzymes and accumulation of this compound leads to increased TAG synthesis and lipid droplet content.

Table 2.1: Kinases of phospholipid synthesis and regulatory proteins

Gene	Encoded enzyme	Phosphorylation enzyme	Phosphorylation site	Result	Ref
CHO1	phosphatidyl serine synthase	Protein kinase A	Ser^{46} and Ser^{47}	Inhibition	(Choi *et al.* 2010)
CKI1	choline kinase	Protein kinase A	Ser^{30} and Ser^{85}	Activation	(Yu *et al.* 2002)
OPI1	Opi1p transcription factor	Protein kinase A	Ser^{31} and Ser^{251}	Activation	(Sreenivas and Carman 2003)
		Protein kinase C	Ser^{26}	Inhibition	(Sreenivas *et al.* 2001)
		Casien kinase II	Ser^{10}	Activation	(Chang and Carman 2006)
PAH1	PA phosphatase	Cyclin-dependant Kinase	Multiple Ser and Thr sites	Inhibition	(O'Hara *et al*. 2006)
URA7	CTP synthetase	Protein kinase A	Ser^{424}	Activation	(Park *et al*. 2003)
		Protein kinase C	Ser^{424}, Ser^{36} and Ser^{354}	Activation	
		Protein kinase C	Ser^{330}	Inhibition	
ACC1	Acetyl-coA carboxylase	Snf1 kinase	Ser^{1157}	Inhibition	(Hofbauer *et al.* 2014)

2.5 Stress

It is of great importance for cells to have the ability adapt to environmental stresses. Since the membrane forms the barrier between the cell and the environment, it plays a vital role in adaptation to many of these changes. Although some stress-specific responses exist, many of the responses overlap between conditions (Beales 2004). Alterations are mainly effected to produce and maintain the correct level of membrane fluidity and curvature as well as to preserve bilayer forming ability (De Kroon *et al*. 2013).

2.5.1 Temperature

Changes in temperature have an impact on membrane fluidity which, in turn, impacts the barrier functioning of the membrane as well as uptake processes (Beales 2004). Increased temperatures may lead to protein denaturation and increased membrane fluidity which can cause leakage of cellular constituents (Balogh *et al.* 2013). It is thought that changes in temperature may be sensed by cells due to alterations in phase state and micro-domain organisation of membrane lipids and

these factors control the activity of the membrane associated proteins which play a role in heat sensing and response (Balogh *et al.* 2013). Changes in proteins due to changes in membrane physical state have previously been described (Escribá *et al*. 2008). However, this may not be the sole mechanism employed against changes in temperature. Some have also found that lipids may influence resistance to heat stress independently of the heat shock proteins through the reduction of membrane fluidity (Swan and Watson 1999).

Decreased temperature results in increased membrane order which eventually leads to phase separation (Guschina and Harwood 2006). Yeast grown in decreased temperatures have been found to increase the abundance of fatty acids with shorter chain lengths in phospholipids as well as decreased saturation which is accomplished through desaturase activity (Beltran *et al.* 2008). The shortening of fatty acyl chains is thought to decrease the amount of carbon-carbon interactions, thus contributing to membrane fluidity (Beales 2004). Likewise, decreased saturation prevents close packing of the phospholipids which leads to increased fluidity and counteracts the effects of temperature reduction (Beltran *et al.* 2008). However, the mechanism behind the control of the fatty acid chain length ratio is unknown. Also, a specific sensor of temperature which initiates alterations in lipid homeostasis has not yet been identified (Balogh *et al*. 2013).

2.5.2 Ethanol

The presence of ethanol, similarly to high temperatures, results in increased membrane permeability (Tierney *et al*. 2005). In a study done by Henderson *et al*. correlation was shown between cellular phospholipid composition and resistance to ethanol in different yeast species (Henderson *et al.* 2013). Here it was seen that cells with low ethanol tolerance tended to have higher content of PI and cells with higher resistance to ethanol showed higher content of PC. PC has previously been found to increase resistance of model membrane systems to the deleterious effects of ethanol (Henderson *et al.* 2013). In further confirmation of these results, a study correlating environmental conditions to gene expression levels also found that extracellular ethanol concentration was correlated to the PI content of cells (Jewett *et al*. 2013).

Early studies on the degree of fatty acid chain saturation indicated that *S. cerevisiae* had increased tolerance to ethanol as the percentage of unsaturated fatty acids increased (Alexandre *et al.* 1994; Mishra and Prasad 1989), a surprising result, given that this is expected to increase membrane fluidity. However, a later study showed that ethanol tolerance was linked more strongly to increased chain length (You *et al.* 2003). This alteration in chain length leads to increased membrane thickness which increases the stability of the membrane and is also thought to be important for maintenance of transport and signal transduction (Henderson *et al*. 2011).

2.5.3 Oxidative stress

It is well known that oxidative stress causes lipid peroxidation which leads to the damage of cellular membranes (Valencak and Azzu 2014). Through the alteration of the physical properties of the membrane, oxidation can also lead to changes in membrane fluidity, protein structure and cell signalling (Bienert *et al.* 2006). The susceptibility of lipids to peroxidation is determined by lipid class, saturation, microdomain organisation and the physical state of the membrane (Catalá 2012). It has been found that membranes with a high percentage of saturated fatty acids are more resistant to oxidative stress than those with a higher degree of unsaturated fatty acids, however this effect was limited to anaerobic conditions (Steels *et al.* 1994). The increased resistance observed could be due to the fact that membrane lipid composition, specifically the length and degree of saturation of the fatty acyl chains influences membrane permeability to oxidants such as hydrogen peroxide (Bienert *et al.* 2006).

In response to exposure to oxidative stress, fatty acid residues affected by peroxidation are cleaved through the action of phospholipase A_2 and replaced (Balboa and Balsinde 2006). Information regarding the effects of oxidative stress on the phospholipid composition of yeast is limited. However, a study in red blood cells showed a decrease in PS and increase in PC in the presence of oxidants and treatment with anti-oxidants resulted in the opposite effect (Freikman *et al.* 2008).

2.5.4 Other

The effects of other stresses on lipid homeostasis have also been tested. However, although much work has been done on this topic in bacteria, not many of these conditions have as yet been tested in yeast. These conditions include; exposure to weak acids, osmotic stress, salt stress and glucose limitation, dehydration and the impact of lipid composition of cell longevity.

Cellular resistance to weak acids is dependent on the control of cells over the permeability of the cell membrane (Beales 2004). Studies in bacteria have found an increase in production of amino-phospholipids (PC, PE and PS) after incubation at a low pH and the authors suggest that the resultant increase in the positive charge of the membrane may aid making the cell impermeable to protons (Beales 2004). A study comparing the resistance of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* to acetic acid stress found that increased levels of sphingolipids and saturated fatty acids played a role in the superior tolerance of the latter to acetic acid (Lindberg et al. 2013). This was attributed to the increased membrane stability and consequently decreased membrane permeability conferred by these changes.

Osmotic stress leads to the formation of a non-bilayer phase within the membrane resulting in reduced membrane function (Beales 2004). In previous studies it has been observed that bacteria

respond to this stress through an increase in anionic phospholipids (phosphatidylglycerol and diphosphatidylglycerol) in comparison the neutral or zwitterionic phospholipids (Hosono 1992).

Dehydration causes damage to cell membranes due to the rapid efflux of water that occurs in this process (Rodríguez-Porrata *et al.* 2011). Yeast species with low levels of viability after rehydration have been found to also contain lower levels of PC (Rodríguez-Porrata *et al.* 2011). In this study it was found that supplementation with PC led to increased levels of this phospholipid in *Saccharomyces paradoxus* and *Saccharomyces bayanus* and resulted in improved viability after rehydration.

A link has also been suggested between the lipid composition of the mitochondrial membrane and cell longevity (Valencak and Azzu 2014). The mitochondria are important in the aging process and their proper functioning key to cell longevity (Valencak and Azzu 2014). Lithocholic acid (LCA), a bile acid known to increase cell longevity was recently used to investigate the mechanism behind this process (Beach *et al.* 2013). LCA was found to accumulate in the mitochondrial membranes and treatment of cells with this compound increased the level of bilayer forming phospholipids relative to the non-bilayer forming phospholipids in this organelle (Beach *et al.* 2013). The mitochondria of cells treated with LCA were also fewer and larger in size and exhibited altered function and morphology (Beach *et al.* 2013). Therefore, this study provided a link between the phospholipid composition of mitochondria and their structure and function in relation to cell longevity.

2.6 Lipidomic studies: Yeast as a model

The field of lipidomics has lately become of great interest due to the many diseases which are thought to include the alteration of cellular lipid composition in either their onset or in their progression. Examples of this include obesity, diabetes and Alzheimer's disease (Santos and Riezman 2012).

Use of *S. cerevisiae* as a model organism for the study of lipid metabolism has many advantages due to the ease of culturing this yeast, its quick generation time, and the variety of molecular tools available for this organism (Zhang *et al.* 2010). *S. cerevisiae,* with its relatively simple lipidome is thought to be a useful model for the study of the lipid synthesis and regulatory pathways, since most of the pathways which are employed in mammalian cells, are also present in yeast (Klose *et al.* 2012). In addition, previous work in yeast has resulted in a good understanding of the localization and regulation of the enzymes involved in lipid metabolism (Huh *et al*. 2003; Jewett *et al*. 2013; Tong *et al.* 2004). These advantages make *S. cerevisiae* and ideal organism in which to screen for links between phenotypes and lipid regulation using the high throughput approaches that are now available (Santos and Riezman 2012). An added benefit is the ability of this organism to grow in defined media which allows for control over the availability of lipid biosynthetic

precursors and other compounds which may influence these processes (Santos and Riezman 2012).

However, there are some differences in lipid metabolism between yeast and mammalian cells which are important to consider when extrapolating results. Yeast only synthesise a limited number of fatty acids relative to mammalian cells and also only desaturate lipids in the Δ9 position (Henry *et al.* 2012). This leads a far smaller diversity of lipid species when compared with mammalian cells where there are thought to be tens of thousands of molecular lipid species (Ejsing *et al*. 2009; Yetukuri *et al.* 2008). Secondly, although mammalian and yeast cells share most of the biosynthetic pathways of phospholipids, some differences do exist. One instance of this is that in yeast CDP-DAG is used for the synthesis of PS, whereas in mammalian cells this phospholipid is produced from PE in an exchange reaction where ethanolamine is replaced with serine (Gaspar *et al*. 2007). Also, while in yeast the CDP-DAG pathway is the preferred method for the synthesis of PC in the absence of supplemented choline; mammalian cells preferentially use the Kennedy pathway to produce PC (Kent and Carman 1999).

However, despite these differences, yeast remains a useful model for the study of lipid metabolism and homeostasis. Significant progress has already been made in the identification of genes involved in the pathogenicity of various diseases with the use of yeast as a model (Auluck *et al.* 2010; Nair *et al.* 2014; Padilla-López *et al*. 2012). Recently, studies in yeast found that fatty acid elongases were important in reducing toxicity of α-synuclein, the protein found to accumulate in Parkinson's disease (Auluck *et al.* 2010). A link between toxicity of the Alzheimer's associated β-amyloid protein and membrane phospholipid composition has also been discovered in yeast (Nair *et al.* 2014). Also, the effects of *BTN1* deletion (*CLN3* in mammals) on lipid composition has been studied in yeast and indicates that this gene, which is defective in Batten disease, resulted in decreased levels of cellular PE (Padilla-López *et al.* 2012). These examples demonstrate the potential of this approach for better understanding of lipid homeostasis in general as well as the usefulness of yeast in identifying possible involvement of these pathways in disease pathogenicity.

2.7 Conclusion

The regulatory pathways which control the synthesis of lipids are at present still poorly understood, and in particular many questions remain regarding the functional diversity of lipid species as well as the mechanisms used by cells to sense and alter their lipid composition (Santos and Riezman 2012). Although studies have found alterations in lipid composition in response to changes in environmental conditions, the sensors and effectors used to accomplish this are largely unknown (Balogh *et al.* 2013). Also, little is known about the mechanisms employed in the cellular trafficking and distribution of lipids (Toulmay and Prinz 2012). The diversity of lipid molecules present in the cell and the vast number of cellular processes affected by changes in lipid metabolism further complicate this research and highlights the need for comprehensive approaches in lipid analysis as

intervention in one pathway can have unknown effects on other lipid classes (Santos and Riezman 2012).

In addition, the lack of standardised extraction protocols and annotation make comparison of currently available information difficult (Santos and Riezman 2012). Relating effects of perturbations in the lipid pathways on the lipidome is also challenging and requires the quantification of lipid species to accurately relate functional genomics to lipidomics, and, in attempts that have been made, lipidomics has seldom been found to complement functional genomics (Ejsing *et al.* 2009). This is due to several reasons including the complex extraction processes, the large sample sizes required and poor availability of appropriate internal standards (Henderson *et al.* 2011).

In conclusion, due to recent advancements in technology the ability to study the lipid composition of cells has increased over the last few years (Santos and Riezman 2012). This has led to a better understanding of many of the mechanisms governing the synthesis and degradation of lipids, particularly in response to changes in environmental conditions. Using yeast as a model, the progress of research in this area may also have a broader application in the better understanding of diseases that include alteration of lipid homeostasis and thus also play a key role development of effective treatments.

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CHAPTER 3: RESULTS AND DISCUSSION

Investigating the effects of carnitine during oxidative stress in *Saccharomyces cerevisiae*: Membrane lipid composition and the relationship with choline

3.1 Introduction

In eukaryotes, L-carnitine has long been studied for its role in the carnitine shuttle where it aids in the transport of activated fatty acyl residues between the mitochondria, peroxisomes and cytosol (Vaz and Wanders 2002). However, recently attention has been diverted to the study of the protective effect of carnitine against oxidative stress (Li *et al*. 2012; Sachan *et al*. 2012; Silva-adaya *et al*. 2008). In previous studies in this laboratory carnitine supplementation resulted in improved growth for cultures stressed with hydrogen peroxide (Franken and Bauer 2010). However, it was also found that carnitine increased the toxic effects of the thiol reducing agent DTT. These effects were found to occur independently of the carnitine shuttle (Franken *et al*. 2008). Although several interactions of carnitine with cytoprotective genes, membranes and membrane associated proteins have been suggested (Arduini *et al*. 1993; Butterfield and Rangachari 1993; Calabrese *et al.* 2006), no biological function that would be unrelated to the carnitine shuttle has thus far been investigated on a molecular level.

Previous work in this laboratory used a genetic approach by screening for mutations that would suppress carnitine related phenotypes. In particular, the yeast gene deletion library (Giaever and Nislow 2014; http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) was used and several mutants which were less responsive to carnitine in combination with either hydrogen peroxide or DTT were identified. Here we focus on the further characterisation of two of these mutants, Δ*opi3* and Δ*cho2*, which code for enzymes that catalyse sequential methylation reactions in the synthesis of phophatidylcholine (PC) from phosphatidylethanolamine (PE) in the CDP-DAG pathway (Figure 3.1) (Henry *et al.* 2012). However, PC can also be synthesised through the Kennedy pathway which requires the uptake of external choline (Henry *et al*. 2012).

PC is a major membrane phospholipid which is thought to account for approximately 50% of the phospholipids within the cell and is thus thought to be important in membrane structure (Boumann *et al*. 2006). With recent advances in technology enabling more precise analysis of lipids, the study of lipid composition, homeostasis and regulation has become of greater interest. In particular, various studies have investigated the regulation of lipid composition under stress conditions and how this influences cell survival (Balogh *et al.* 2013; Beales 2004; Henderson *et al*. 2013).

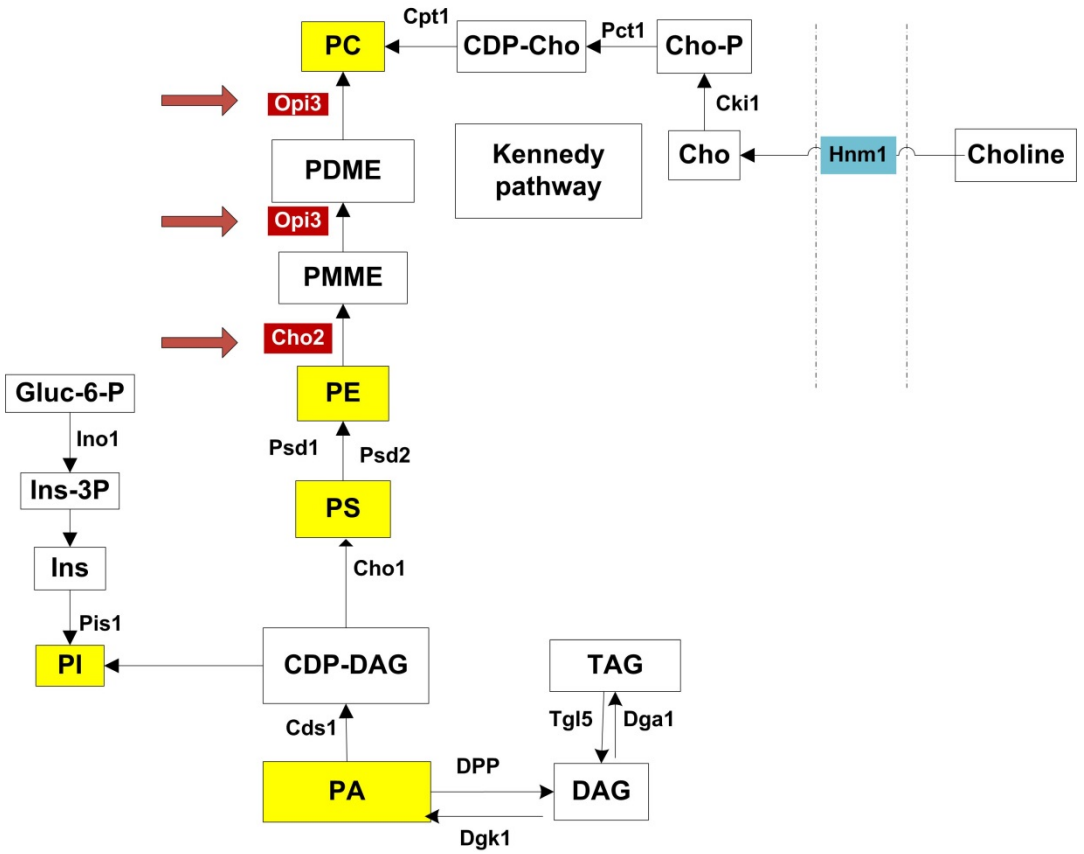

Figure 3.1: Phospholipid synthesis pathways. All major phospholipids and TAG are highlighted in yellow. Opi3p and Cho2p, highlighted in red, catalyse methylation reactions in the synthesis of PC from PE. PC may also be synthesised via the Kennedy pathway when external choline is taken up through the transporter Hnm1p. Abbreviations: PA, phosphatidic acid; DAG, diacylglycerols; TAG, triacylglycerols; CDP-DAG, CDP-diacylglycerol; PI, phosphatidylinositol; Ins, inositol; Ins-3P, inositol 3-phosphate; Gluc-6-P, glucose-6-phosphate; PS, phosphatidylserine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyl-dimethylethanolamine; PC, phosphatidylcholine; CDP-cho, CDP-choline; Cho-P, choline phosphate; Cho, choline. Adapted from (Henry et al. 2012).

The genetic link between the carnitine-dependent phenotypes and suppressor mutations in genes involved in PC synthesis suggests that carnitine may impact membrane lipid composition or homeostasis. For this reason, the relationship between choline and the phenotypes associated with carnitine supplementation was studied. In addition, the link between the carnitine-dependent phenotypes and the lipidome of cells was also investigated through analysis of the effects of deletion of *OPI3* and *CHO2* as well as treatment with carnitine, choline, hydrogen peroxide and DTT on the lipid composition of cells.

3.2 Materials and methods

3.2.1 Strains and media

The strains used in this study were obtained from Euroscarf (Frankfurt, Germany) and are listed in table 3.1. Strains were grown in minimal SCD media (2% glucose, 0.67% YNB without amino acids

(Difco)). Amino acids were supplemented as required by the different strains at a concentration previously described (Ausubel *et al*. 1994). The DTT, carnitine and choline used in this study were obtained from Sigma.

Table 3.1: Strains used in this study

Yeast strains	Genotype	Ref
BY4742	MATα *his3 lys2 leu2 ura3*	Euroscarf
BY4742Δ*opi3*	MATα *his3 lys2 leu2 ura3*Δ*opi3*::kanMX	Euroscarf
BY4742Δ*cho2*	MATα *his3 lys2 leu2 ura3*Δ*cho2*::kanMX	Euroscarf

3.2.2 Spotting assay

Media was made up on the day prior to analysis and 8 mM DTT or 1 mM hydrogen peroxide with or without 5 mM carnitine was added where appropriate as previously described (Franken and Bauer 2010). Strains were grown overnight at 30°C in 5 ml SCD. Cultures were then diluted to OD_{600nm} of 1 and five sequential ten times dilutions were made in a microtitre plate. These were then spotted onto plates in 10 µl volumes. Plates were incubated at 30°C.

3.2.3 Liquid growth assays

Strains were grown in 5 ml SCD overnight at 30°C and then inoculated into a second round of starter cultures in 10 ml volumes and grown overnight. The cultures were then spun down and resuspended in 10 ml medium with and without carnitine and/or choline at a final OD_{600nm} of 0.1. Cultures were grown until mid-log phase and then treated with hydrogen peroxide or DTT. The optical density of the cultures was monitored over a period of 30 hours. For survival assays, cultures were plated out just before and for several time points after addition of hydrogen peroxide.

3.2.4 Lipid extraction

Prior to lipid extraction cultures were grown to an OD_{600nm} of 0.8 unless otherwise specified and then treated with DTT or hydrogen peroxide. The cultures were then spun down at 6000 RPM for one minute, washed with distilled water, weighed and frozen at -80°C. Lipid extraction was carried out as previously described (Ejsing *et al*. 2009) with some alterations. Cultures were thawed prior to extraction, resuspended in 200 µL NH_4HCO_3 (150 mM, pH 8) and 300 µL glass beads were added. Cells were disrupted by vortexing for three minutes. Internal standards were then added for; PI (40 mg/L), PS (12 mg/L), PA (12 mg/L), PE (4 mg/L) and PC (4 mg/L). For the first extraction, 1 ml chloroform: methanol (17:1) was added and samples were placed on a shaker for two hours at 30 °C. The organic phase was then removed and placed in a new tube and the aqueous phase was re-extracted with 1 ml chloroform: methanol (2:1) and 0.5% formic acid which was again carried out on a shaker at 30 °C for two hours. Thereafter, the organic phase was

collected and added to the first. Samples were then dried, resuspended in chloroform: methanol (1:2) and stored at -20 until analysis. The chloroform, methanol and formic acid used were obtained from Sigma.

The internal standards used for lipid analysis were: 1,2-dilauroyl-sn-glycero-3-phosphate (PA 12:0-12:0), 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine (PE 18:2-18:2), 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (PS 18:2-18:2), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (PC 18:2-18:2) and L-α-phosphatidylinositol (liver, bovine) (PI 18:0-20:4). The reference standards used were: 1,2-diheptadecanoyl-sn-glycero-3-phosphate (PA 17:0-17:0), 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE 17:0-17:0), 1,2-diheptadecanoyl-sn-glycero-3-phospho-L-serine (PS 17:0-17:0), 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (PC 15:0-15:0) and L- α-Phosphatidylinositol (soy) (PI 16:0-18:2). The lipid standards were obtained from Avanti Polar lipids.

3.2.5 HILIC-ESI-MS

The analysis of the lipid composition of cultures was adapted from a method developed by Castro-Perez and co-workers (Castro-perez *et al*. 2010). Analysis was performed on a Waters Acquity UPLC (Waters, Milford, MA, USA), connected to a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer fitted with an electrospray ionisation probe. Compounds were separated on a Waters HSS T3 column, 2.1x150 mm, with a 1.8 µm particle size at a flow rate of 0.32 ml/min. The injection volume was 5 µl. Lipids were analysed both in positive (PC, PE, TAG) and negative (PI, PS, PA) mode using a capillary voltage of 2.5kV, cone voltage of 15V, desolvation gas (nitrogen) flow rate of 650L/hr and temperature at 275°C. Solvent A was made up of 0.6 g/L ammonium formate in 60% acetonitrile and solvent B consisted of isopropanol-acetonitrile (9:1) with 0.6 g/L ammonium formate. For the first 11 minutes of the run 60% solvent A was used. Thereafter, this was changed to 100% solvent B. At 14 minutes it was returned to 60% A. The total run time was 17 minutes. Data was acquired in MS^E mode which consists of a lower collision energy scan (*m/z* 150-1500 at 6 V) and a higher energy scan (*m/z* 40 to 1500, collision energy ramp from 20 to 60V) every 0.2 seconds. The instrument was calibrated with sodium formate and leucine enkephalin was used as lock spray for accurate mass determinations. The resulting data was analysed using Masslynx version 4.1.

3.2.6 Statistical analysis

All data are shown as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using Student's t-test and $p < 0.05$ was considered significant and $p < 0.01$ and 0.001 very significant.

3.3 Results

3.3.1 Δ*cho2* and Δ*opi3* phenotypes

Wildtype and mutant strains were grown on solid media containing hydrogen peroxide or DTT in the presence or absence of carnitine. The assays showed reduced growth for all strains on plates containing 1 mM hydrogen peroxide (Figure 3.2). However, in the presence of 5 mM carnitine, a protective effect is apparent for the wild type, but not in the two mutants. The addition of 8 mM DTT to media also resulted in decreased growth in all strains. The data show that growth of the WT is further reduced upon co-supplementation of carnitine and DTT, but that this does not apply to Δ*cho2* or Δ*opi3*.

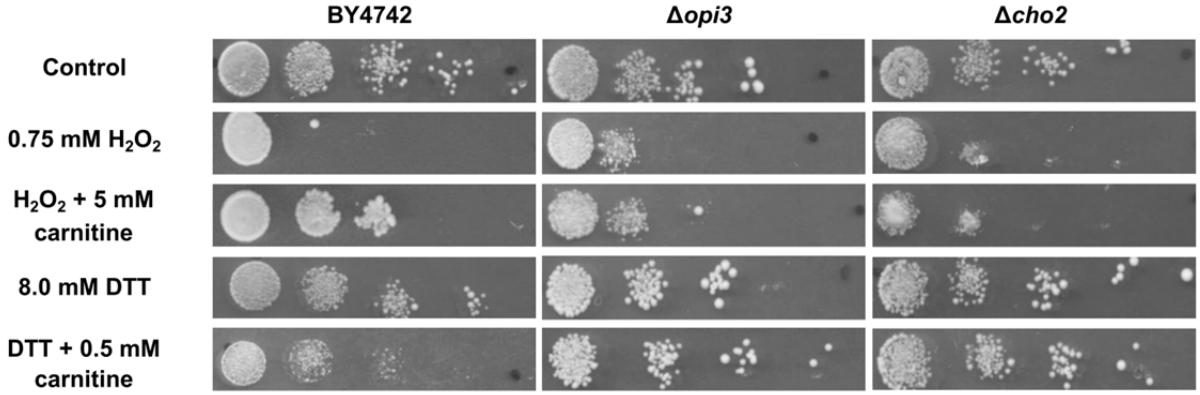

Figure 3.2: Plate assays for response of wild type BY4742 and the mutants; Δ*cho2* and Δ*opi3* when stressed with DTT or hydrogen peroxide in the presence and absence of carnitine.

The inability of the mutants which are involved in the synthesis of phosphatidylcholine to respond to carntine treatment suggests a link between the observed carnitine-dependent phenotype and choline. Carnitine and choline share a striking similarity on a structural chemical level, both containing a trimethylammonium moiety, and also make use of at least one shared uptake mechanism through the transporter Hmn1p (Aouida *et al*. 2013). To further analyse the suppression of carnitine-dependent phenotypes by the two mutants, similar experiments were carried out in liquid medium in order to obtain a clearer distinction between the phenotypes. For this purpose the concentrations of carnitine, DTT and hydrogen peroxide, as well as the timing of treatment with the redox stress inducing chemicals, were optimised.

Addition of hydrogen peroxide to the wild type cultures inhibited growth for a period of time after which cultures resumed growth. Cultures supplemented with carnitine recovered faster than those without carnitine (Figure 3.3 A). This effect was dependent on the concentration of carnitine added, with 0.05 and 0.5 mM providing no positive effects and the maximum response reached at 2.5 mM carnitine. In addition, the plate counts show that cultures containing carnitine had a higher percentage survival than those without carnitine (Figure 3.3 C).

The treatment of cultures with DTT resulted in cessation of growth from which the cells did not recover within the time period monitored (Figure 3.3 B). Cultures which contained carnitine in addition to DTT plateaued at a lower optical density than those with only DTT. The effect for carnitine, as with hydrogen peroxide, occurred in a concentration dependent manner. However, an effect for carnitine could be seen at a lower concentration, namely 0.5 mM, than with hydrogen peroxide.

In order to investigate whether carnitine supplementation had any effect on the growth of cultures, and also to determine whether it is capable of compensating for the loss of choline within the mutants, the wild type and mutant cultures were inoculated into media with and without 2.5 mM carnitine (Figure 3.4). It was found that wild type cultures grew slightly slower in the presence of carnitine in the early stages of growth but caught up with unsupplemented cultures towards the end of the log phase. However, carnitine did not compensate for loss of choline in the mutant cultures. The growth-impeding effect of carnitine was even stronger in Δ*opi3* than the wild type. However, carnitine supplementation had no significant effect on the growth of Δ*cho2.*

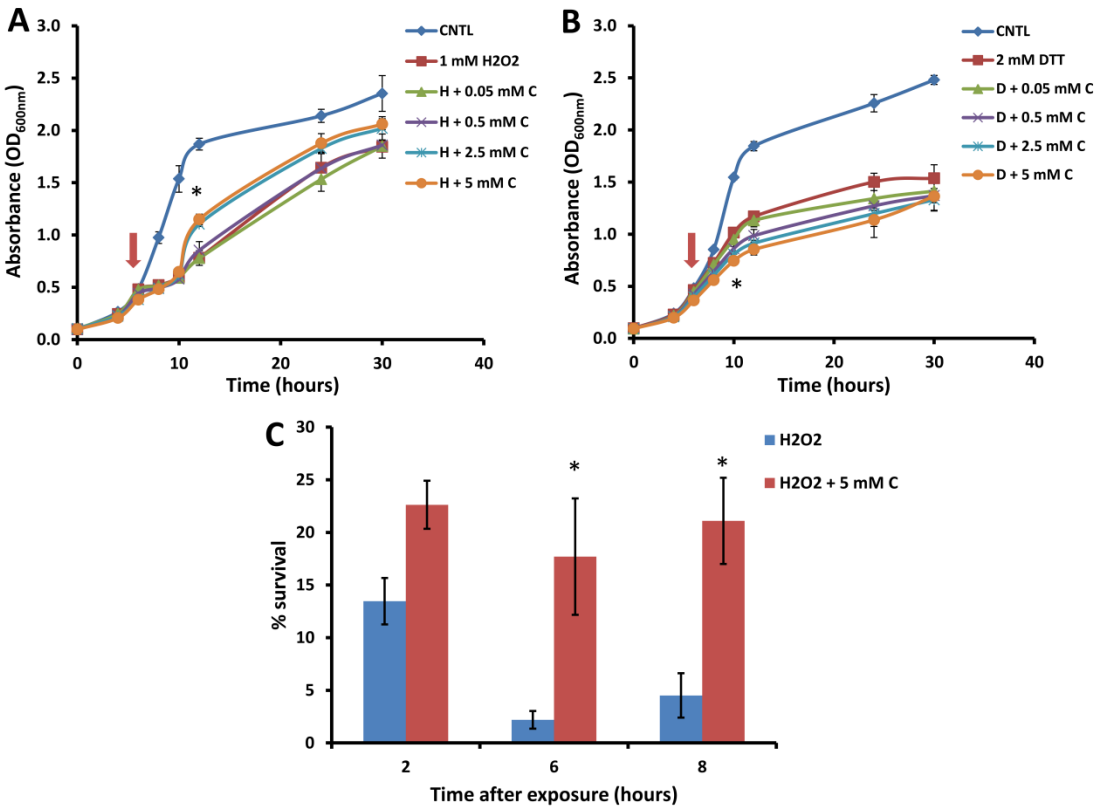

Figure 3.3: Response of BY4742 strain to oxidative stress induced by hydrogen peroxide and DTT in the presence and absence of carnitine (C). A: Liquid analysis monitoring growth before and after exposure to hydrogen peroxide. B: Liquid analysis monitoring growth before and after exposure to DTT. The time at which either DTT or hydrogen peroxide was added is indicated by an arrow. C: Percentage survival of cultures grown in the presence or absence of carnitine after exposure to hydrogen peroxide. All experiments were done in triplicate with error bars representing standard deviation (*$p<0.05$; 5 mM carnitine vs. H_2O_2 or DTT).

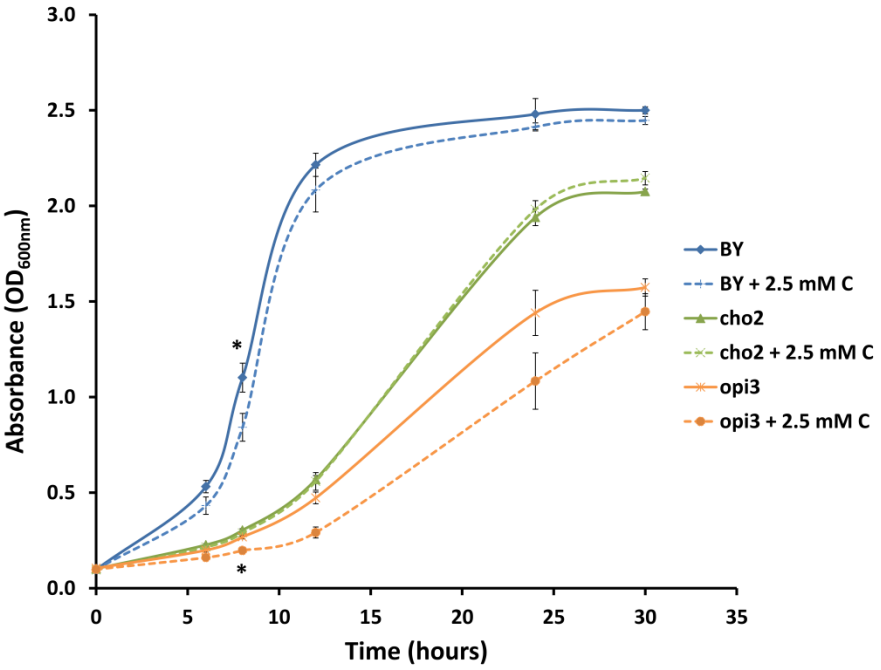

Figure 3.4: Carnitine supplementation (C) led to a slowing of growth of wild type (BY) and Δ*opi3* cultures but not for Δ*cho2*. All experiments were done in triplicate with error bars representing standard deviation (*p<0.05 vs. untreated culture).

3.3.2 Effect of choline addition on carnitine phenotypes

In addition to the synthesis of PC via the CDP-DAG pathway, this phospholipid may also be produced through the Kennedy pathway when cells are supplemented with choline (Dowd *et al.* 2001). It was therefore tested whether choline supplementation to Δ*cho2* and Δ*opi3* mutant cells would restore the effects of carnitine. It was also of interest to establish if choline supplementation to wild type cells would simulate similar phenotypes to those linked with carnitine supplementation.

Addition of choline by itself had no effect on the growth of the wild type cultures (Figure 3.5). There was a slight improvement in recovery time for cultures containing choline in the presence of hydrogen peroxide; however this effect was far less than that observed for carnitine (Figure 3.5 A). In addition, when carnitine and choline were added together at half the concentration than when supplemented alone, the protective effect did not match that of carnitine by itself at the higher concentration. These observations were further confirmed since choline, supplemented alone or in combination with carnitine also did not have any significant effect on the DTT phenotypes (Figure 3.5 B).

These conditions were then also tested in the mutants. Both mutants responded similarly to hydrogen peroxide and DTT as the wild type, however, Δ*cho2* required a higher concentration of hydrogen peroxide than Δ*opi3* or the wild type (2 mM and opposed to 1 mM) for a significant decrease in growth to be observed (Figure 3.5). The reason for the increased resistance in this strain is uncertain. No protective effect for carnitine was observed for Δ*cho2* and Δ*opi3* when stressed with hydrogen peroxide (Figure 3.5 C and E).

As expected, the addition of choline significantly improved the growth of both mutants. Moreover, when choline was present, the protective effect of carnitine in the presence of hydrogen peroxide was restored to WT levels in the case of both phospholipid synthesis mutants.

Both mutants also responded similarly to the wild type when stressed with DTT (Figure 3.5 D and F). For Δ*cho2*, there was no significant increase in toxicity of DTT when applied in combination with carnitine (Figure 3.5 D). This effect of carnitine was, however, restored when choline was also present in the medium. Conversely, in the Δ*opi3* mutant, the effect of DTT was increased in the presence of carnitine regardless of the presence of choline (Figure 3.5 F).

3.3.3 LCMS analysis of lipid composition

3.3.3.1 Analysis of lipid composition

Carnitine is well known for its role in shuttling activated acetyl-coA residues within the cell (Vaz and Wanders 2002). In addition to this, a link between carnitine and phosphatidylcholine biosynthesis has been identified since Δ*cho2* and Δ*opi3* were found to suppress the carnitine phenotypes. To further investigate the connection between phospholipid biosynthesis and the carnitine phenotypes, the lipid composition of the wild type was compared with that of Δ*cho2* and Δ*opi3*. In addition, the effect of treatment with choline, carnitine, H_2O_2 and DTT on the lipid composition of cells was also analysed.

The quantification of phospholipid concentrations through the use of standards did not yield repeatable results when comparing different runs. Therefore, the intensity%, which is calculated as the area of the peak of a given compound divided by the total peak area of all the compounds in the chromatogram, was used to compare strains and treatments. The results therefore do not represent changes in the concentration of compounds but in the relative abundance of the compound in the sample. This measure was reported in previous studies to have a reliable and linear relationship to the molar abundance of a compound in a sample (Tarasov *et al.* 2014).

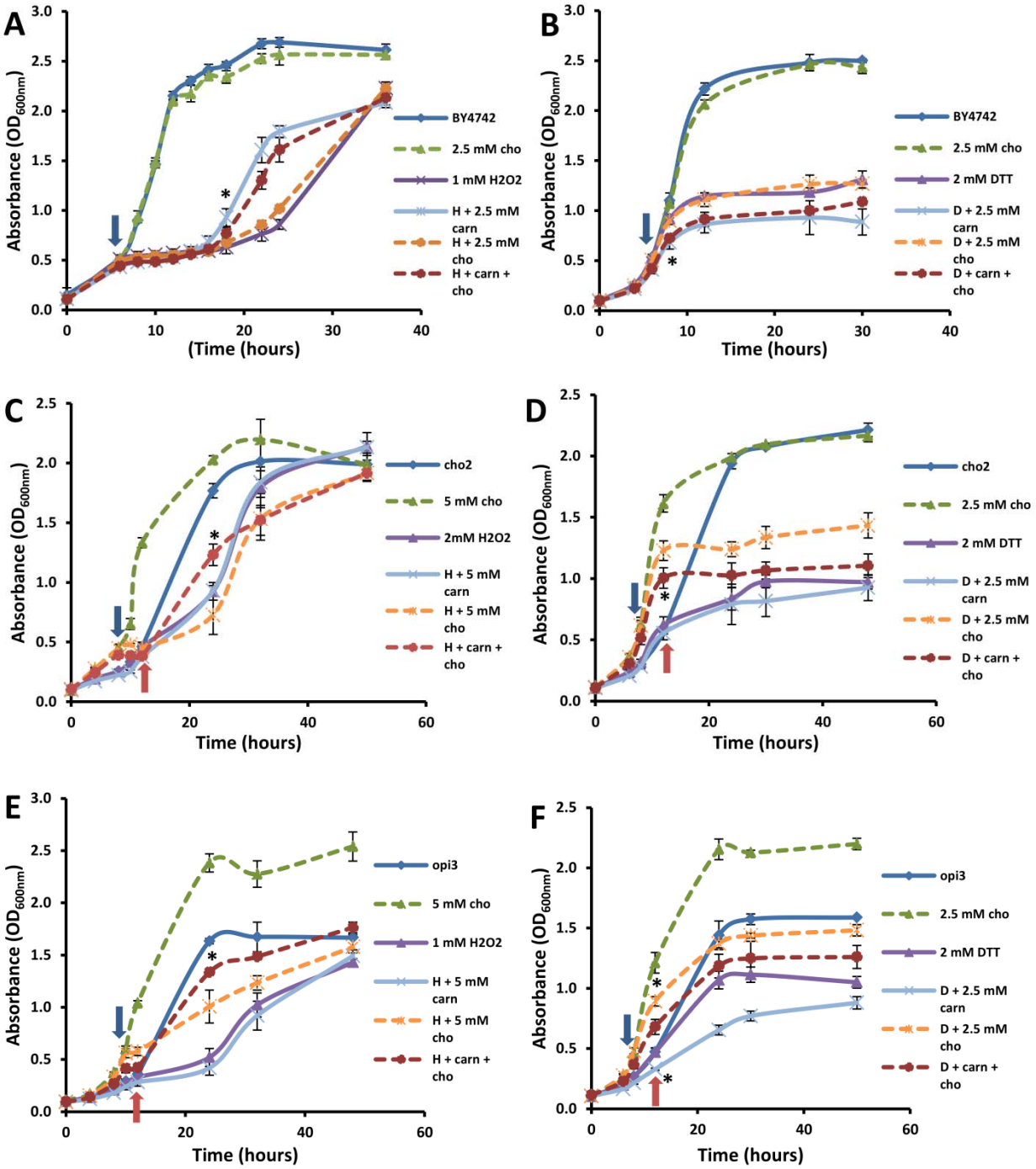

Figure 3.5: Effect of choline on carnitine phenotypes in the mutant and wild type cells. Broken lines indicate choline-treated cultures. Addition of choline (cho) has no significant effect alone or in combination with carnitine (carn) on either hydrogen peroxide (A) or DTT (B) in the WT. Choline supplementation restores the effects of carnitine in the mutant Δ*cho2* both in hydrogen peroxide (C) and DTT (D). The presence of choline also restores the effect of carnitine in for Δ*opi3* hydrogen peroxide (E). However, under these conditions Δ*opi3* was found to be responsive to carnitine together with DTT, irrespective of the presence of choline (F). Arrows represent the timing of addition of DTT or hydrogen peroxide. For the mutants blue arrows indicate the timing of treatment for cultures grown with choline and red arrows for cultures grown without choline. All experiments were done in triplicate with error bars representing standard deviation (*$p<0.05$ vs. H_2O_2 or DTT).

3.3.3.2 Comparison of the phospholipid composition between the wild type and Δ*opi3* in different growth stages

To compare the phospholipid composition of the wild-type with Δ*opi3* at different growth stages and also to examine the effect of choline depletion on the lipidome of cells, both strains were pre-cultured in medium containing choline and then transferred to medium without choline. The cultures were then sampled at four different growth stages over a period of 30 hours (Figure 3.6). As would be expected, the abundance of PC in the mutant culture decreases. The abundance of PE in both strains also decreases as the cultures approached stationary phase. For both the wild type and mutant cultures, the levels of TAG increase towards stationary phase, however, the levels of TAG present in the mutant were always significantly higher than in the wild type reaching up to three fold higher at the end of log phase. PI was also present in significantly higher levels in the mutant than in the wild type at stationary phase with a 40% difference in abundance. An increase in PA was seen for both strains as cultures reached stationary phase and the opposite trend was observed for PS, although this was much more prominent in the mutant than the wild type.

The fatty acid distribution within the phospholipids was also analysed (Figure 3.7). In yeast these are primarily composed of 32:1, 32:2, 34:1, 34:2 and 36:1, which are made up of C16:0, C16:1, C18:0 and C18:1 acyl chains esterified to the *sn1* and *sn2* positions on the glycerol backbone (Boumann *et al.* 2006). Compared to the wild type, Δ*opi3* displayed decreased abundance of 32:1 and 34:2 fatty acid chains of 33% and 8%, respectively within PC and PE by stationary phase, while the levels of these fatty acids were not much altered within PI, PS and PA. The mutant also displayed increased abundance of 23% in 32:2 within PC and PE and 16% in 36:1 within PI, PA and PS by stationary phase.

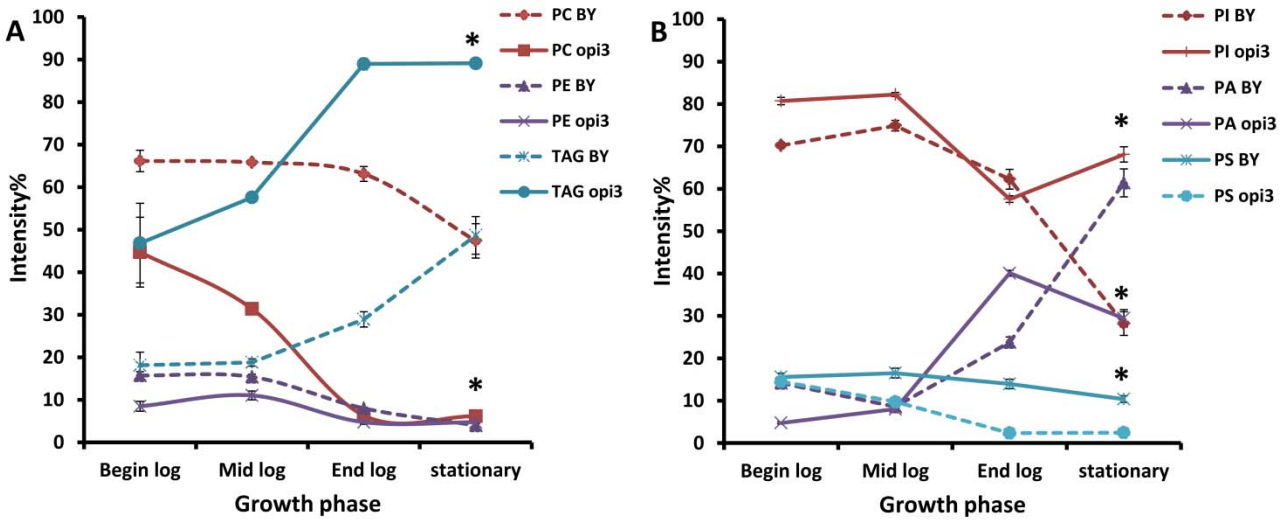

Figure 3.6: Comparison of the phospholipid and TAG composition of the wild type (BY) and mutant Δ*opi3* cultures during different growth phases. All experiments were done in triplicate with error bars representing standard deviation (*p<0.01 vs. BY).

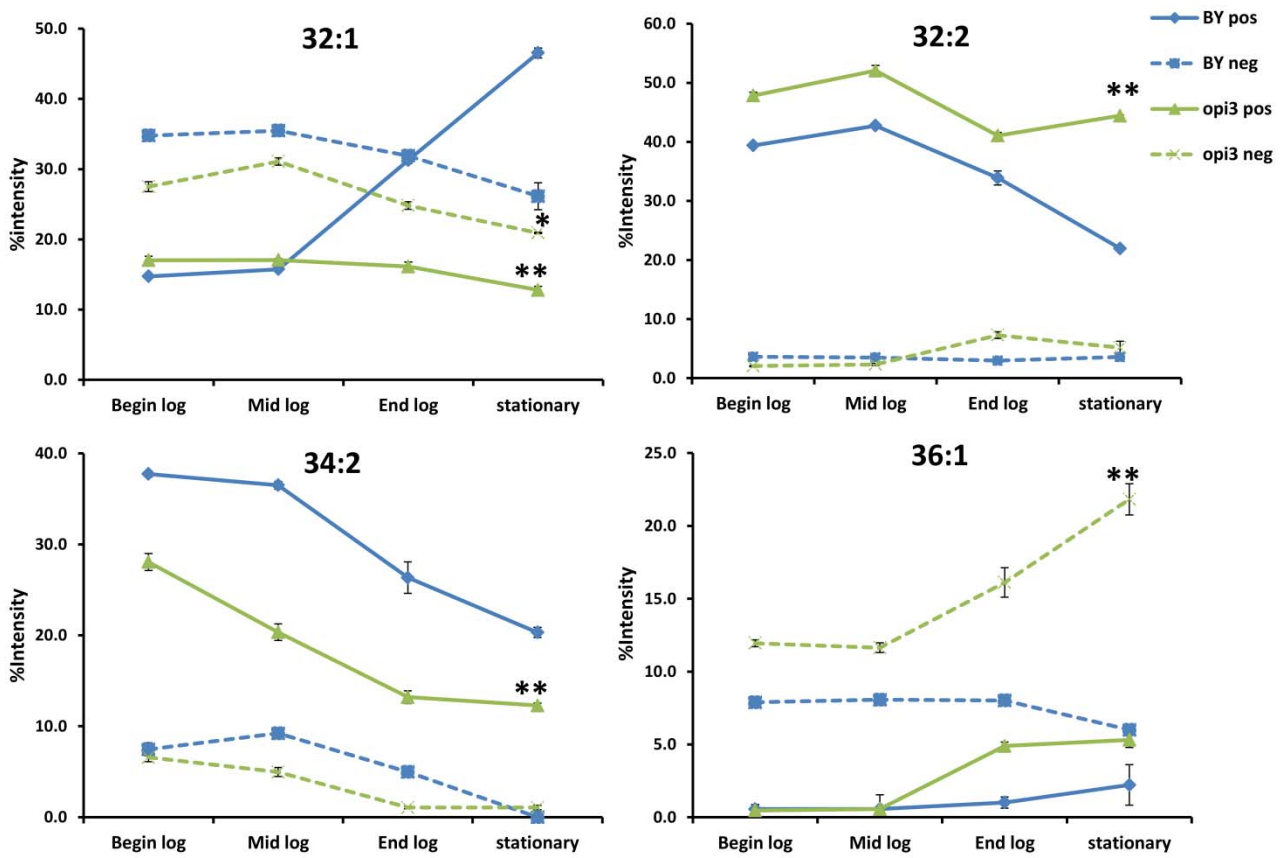

Figure 3.7: Fatty acid distribution of the wild type in comparison with Δ*opi3* at different growth phases. Broken lines indicate the values obtained from the negative ionisation mode and represent the fatty acid profile within PI, PS and PA. Solid lines indicate data obtained from positive ionisation mode and the fatty acids present in PC and PE. All experiments were done in triplicate with error bars representing standard deviation (*p<0.05, **p<0.01 vs. BY).

3.3.3.3 Effect of choline and carnitine supplementation on lipid composition of wild type and mutant cells

To investigate the effects of choline and carnitine supplementation on the lipidome of wild type and mutant strains, cultures were grown in minimal medium supplemented with choline and then transferred to medium which was without or with either 2 mM choline or 2.5 mM carnitine. Samples were taken for lipid analysis at end log phase.

The mutant Δ*cho2* showed a reduction in abundance of PC by 36% and also contained a 34% increase of TAG compared with the wild type (Figure 3.8). Addition of choline led to a reversal of these effects in the mutant, producing a lipid profile similar to that of wild type. Preliminary results also indicate that Δ*opi3* reacts similarly to Δ*cho2* in response to choline supplementation (data not shown). However, the addition of carnitine did not lead to any significant changes in the phospholipid composition of cells.

Deletion of *CHO2* was observed to lead to a similar fatty acid distribution profile as Δ*opi3* (Figure 3.9). The addition of choline led to a fatty acid profile similar to that of the wild type, particularly to increased abundance of 34:2 and decreased abundance of 32:2 fatty acid chains. Carnitine

supplementation did not result in any significant changes in the fatty acid profile of the mutant or wild type cultures.

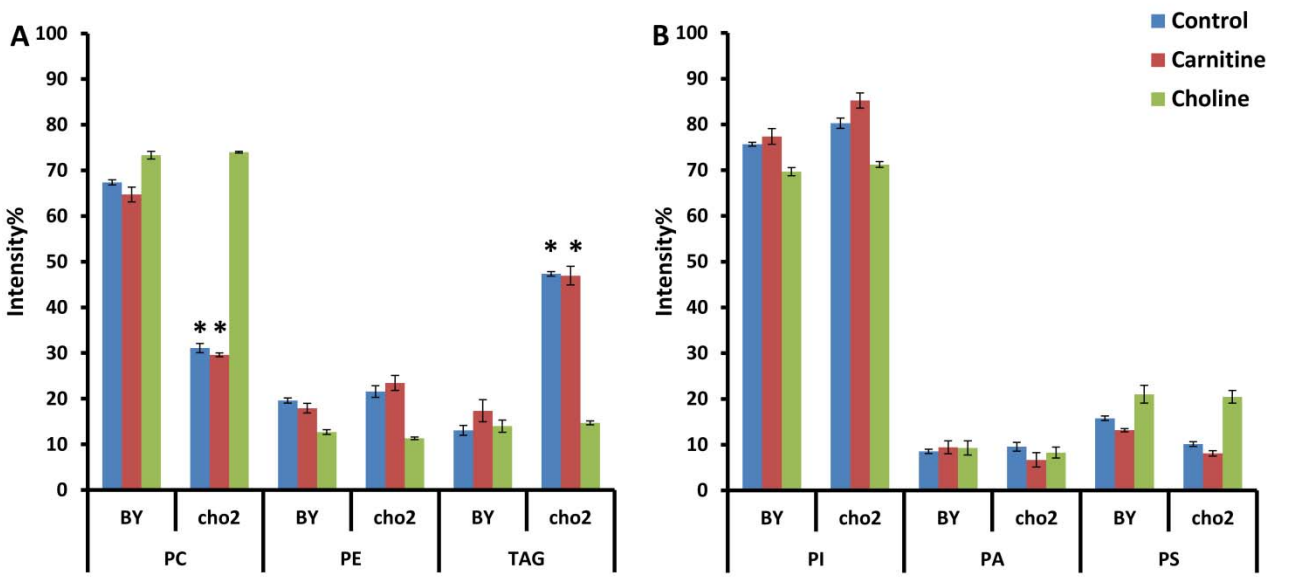

Figure 3.8: Phospholipid and TAG composition of the mutants alone and in response to carnitine and choline. All experiments were done in triplicate with error bars representing standard deviation (*p<0.001 vs. BY control).

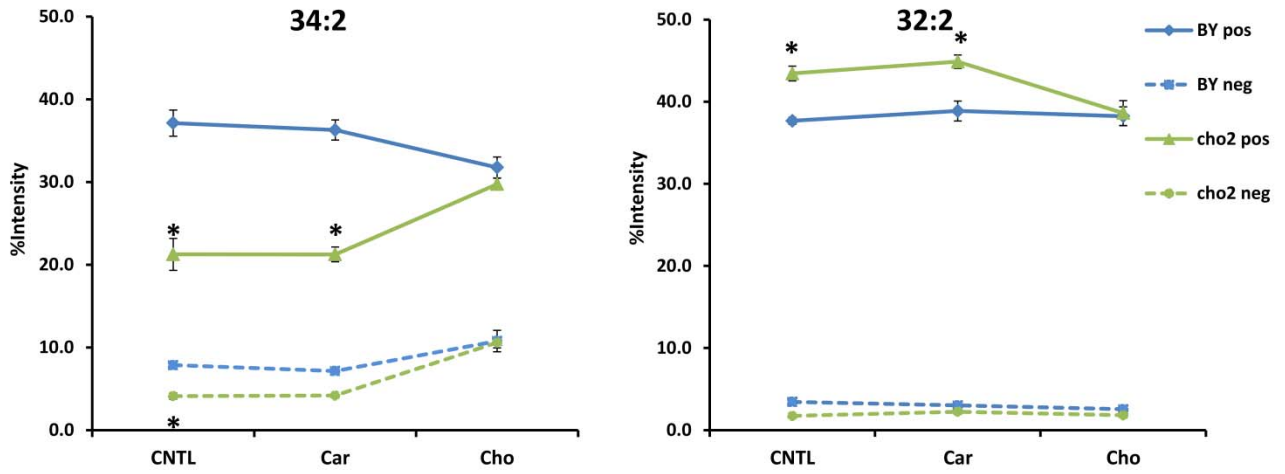

Figure 3.9: Fatty acid distribution of wild type and Δ*cho2* cultures under control conditions (CNTL) and when supplemented with carnitine (Car) or choline (Cho). Broken lines indicate the values obtained from the negative ionisation mode and represent the fatty acid profile within PI, PS and PA. Solid lines indicate data obtained from positive ionisation mode and the fatty acids present in PC and PE. All experiments were done in triplicate with error bars representing standard deviation (*p<0.01 vs. BY control).

3.3.3.4 Effect of treatment with hydrogen peroxide and DTT on lipid composition of cells

It was also investigated whether carnitine treatment results in any changes in the lipid composition of cells after stressing with DTT and hydrogen peroxide. To this end, cultures were grown to an OD_{600nm} of 0.8 in the presence and absence of carnitine, at which point samples were taken for lipid

extraction. Cultures were treated with either DTT or hydrogen peroxide and samples were taken for lipid extraction 30 and 60 minutes after treatment.

Treatment with carnitine by itself did not result in any major shifts in the phospholipid and TAG composition within the cells. Also, no major changes in lipid composition could be seen for cultures treated with hydrogen peroxide or DTT (data not shown).

3.4 Discussion

Although the protective effect for carnitine against oxidative stress has been established by several studies (Franken and Bauer 2010; Li *et al*. 2012; Sachan *et al.* 2012), the mechanism behind this action remains unknown. However, several mutants identified in this work which suppress the carnitine-dependent phenotypes, provide some indication as to the processes likely to be involved in this response. The identification of genes which code for enzymes that catalyse sequential reactions in phosphotidylcholine biosynthesis, Δ*cho2* and Δ*opi3*, strongly suggests the involvement of this pathway or the product of this pathway in the observed phenotypes.

The data confirm the protective effect for carnitine against hydrogen peroxide, and a detrimental effect when carnitine is combined with DTT (Franken and Bauer 2010). Here we identified genes that, when deleted, suppress these effects that carnitine yielded. Deletion in two genes encoding enzymes involved in phosphatidylcholine biosynthesis, *CHO2* and *OPI3,* were identified in this manner. To further investigate the nature of the genetic link between phosphatidylcholine biosynthesis and carnitine supplementation, the effect of choline on the previously observed phenotypes was tested.

In the wild type, choline supplementation alone did not have similar effects to carnitine when combined with hydrogen peroxide or DTT. Also, choline could not compensate for lower concentrations of carnitine under these conditions. Therefore, it is unlikely that the link between carnitine and choline, established through the mutant phenotypes, is as a result of a shared role for these two compounds. The improved growth of the mutant strains in the presence of choline, which restores the synthesis of PC through the Kennedy pathway is as expected (Dowd *et al.* 2001). In addition, the restoration of the protective effect for carnitine against hydrogen peroxide in mutant cultures supplemented with choline indicates that the presence of this compound or phosphatidylcholine is required for this phenotype.

When the mutants were treated with DTT, it was found that Δ*cho2* was unresponsive to carnitine supplementation, except in the presence of choline. However, Δ*opi3* reacted as the WT to the combination of DTT and carnitine. This result was contradictory to what was found on plate assays for Δ*opi3*.

The differences in the results obtained for carnitine and DTT obtained on solid and liquid medium could indicate a possible difference in the responses of cells to DTT and carnitine under these different conditions. Furthermore, it is possible that the growth slowing effect of carnitine could, at least partially, be responsible for the increased toxicity observed when carnitine is supplemented in addition to DTT in liquid media. However, this growth-slowing effect for carnitine does not appear to play a role in the phenotypes observed together with hydrogen peroxide as the reaction of cultures to this ROS inducing agent appears to be independent of the response to carnitine prior to treatment.

Due to the link found between the carnitine shuttle-independent phenotypes and Δ*cho2* and Δ*opi3*, changes in cellular lipid composition in the mutants and in the wild type in response to the different treatments were investigated. It has already been suggested that the composition of the cell membrane could influence the susceptibility of the cell to adverse environmental conditions (Henderson *et al.* 2013; Rodríguez-Porrata *et al*. 2011). Thus it was investigated whether the carnitine phenotypes could be attributed to changes in the phospholipid composition of cells.

To test the effects of choline depletion on the lipidome of cells, wild type and Δ*opi3* cultures grown in pre-cultures containing choline, were placed in media without choline. A decrease in PC abundance in the mutant cultures was observed and became more prominent as biomass increased. The data show that the progressive decline in the availability of this major phospholipid resulted in increased PI and TAG abundance, while the fatty acid chains used in phospholipids underwent changes which included decreased abundance of 32:1 and 34:2 fatty acid chains in favour of 32:2 and 36:1 containing phospholipids. These changes in phospholipid composition had been reported previously and are thought to aid in the maintenance of proper membrane structure and function by decreasing the contribution of PE to negative membrane curvature in the absence of PC (Boumann *et al*. 2006).

The data shows that Δ*cho2* has a similar phospholipid, TAG and fatty acid composition as Δ*opi3*. Addition of choline to mutant cultures restored PC synthesis and led to cultures with phospholipid and TAG profiles much like the wild type. This would indicate that the differences in phospholipid composition observed between the mutant and wild type cultures are due to the associated regulatory changes made in order to maintain proper membrane function in response to decreased PC and are not dependant on a functional CDP-DAG pathway. Previous studies have shown that although the Kennedy and CDP-DAG pathway produce PC with different fatty acyl chains, mutants of either pathway are still able to produce a PC species profile similar to that of the wild type (Boumann *et al*. 2003). This finding emphasises both the importance of a specific species profile for phospholipids and the key role played by post synthetic remodelling in achieving this.

The mutants used in this study have been described as displaying the Opi$^-$ phenotype which is characterised by overproduction of inositol due to overexpression of *INO1* (Mcgraw and Henry

1989; Summers *et al.* 1988). Increased availability of inositol then leads to higher production of PI (Klig *et al.* 1985), which is consistent with the relative abundance of phospholipids found in this study. An increased amount of the PA also appears to be channelled into synthesis of TAG in the mutants. Studies have also shown that deletion of *CHO2* and *OPI3* leads to the accumulation of super-sized lipid droplets (Fei *et al*. 2011) a finding which is consistent with the increased TAG abundance observed in these strains. One of the major functions of TAG within the cell is in storage fatty acids, but recent evidence suggests that this role is, at least partially shared by PC (De Smet *et al.* 2013). In this study, cells were found to accumulate excess palmitate in PC. Thus, in the absence of the ability to synthesise PC, excess fatty acids would need to be stored to a greater extent in the form of TAGs.

Treatment with DTT and hydrogen peroxide did not to lead to changes in the lipid profile of cultures. This is in contrast to results from a study in red blood cells indicated that oxidative stress induced decreases in PS and increases in PC (Freikman *et al*. 2008). However, this is thought to be a strategy to avoid phagocytosis and would therefore not be expected to be employed by yeast. In addition, carnitine did not induce changes in the lipid profile in the wild type or mutant cells and no effect for carnitine supplementation on the lipid composition of cells stressed with hydrogen peroxide or DTT was found. This indicates that alteration of lipid composition is likely not linked to the phenotypic effects of carnitine supplementation. However, it is possible that the presence of PC within the cell membranes is necessary for the carnitine phenotypes.

In conclusion, it was observed in this work that deletion of *CHO2* and *OPI3* led to decreased responsiveness to carnitine supplementation in combination with hydrogen peroxide and also together with DTT with the exception of Δ*opi3* in liquid cultures, where this strain reacted similarly to the wild type. This suggests that cellular processes affected by mutations in these genes are likely involved in the mechanism underlying the action of carnitine. The observation that supplementation with choline restores the effects of carnitine shows that this compound is required for the carnitine phenotypes.

In addition, it is clear from these results that the deletion of the genes encoding for the methyltransferases which are involved in the synthesis of PC from PE result in wide spread changes within the lipidome. However, deletion of *CHO2* and *OPI3* could also lead to other changes within the cell which have not been considered in this work. The specific changes or combination of changes which lead to the decreased responsiveness of these strains to carnitine remains unknown. However, the fact that deletion of these two enzymes, to the exclusion of others which may lead to one or more similar effects, for example, other mutants displaying the opi$^-$ phenotype, but do not display decreased responsiveness to carnitine strongly argue that loss of phosphatidylcholine is in some way involved in these effects. However, the loss of PC results in large-scale alterations in cellular lipid composition any one or combination of which could be

involved in this response. Therefore, further investigation is required to determine the mechanisms behind the action of carnitine and the cellular processes involved in this response.

3.5 References

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CHAPTER 4: RESEARCH RESULTS

Investigating the impact of carnitine supplementation on toxicity induced by oxidative stress in mammalian cell cultures

4.1 Introduction

L-carnitine is a quaternary ammonium compound (Figure 4.1) which is primarily taken up through the diet (Virmani and Binienda 2004). However, minor amounts are also synthesised from the amino acids lysine and methionine (Virmani and Binienda 2004). The primary organ of synthesis is the liver though small amounts of carnitine are also synthesised in the kidney (Hoppel and Davis 1986). Carnitine has been widely studied due to its role in the transport of fatty-acyl residues between the mitochondria, peroxisomes and cytosol in a process known as the carnitine shuttle (Figure 4.2) (Vaz and Wanders 2002). In this process, carnitine is bound to activated fatty acyl residues via the action of carnitine acetyl transferases and translocated across the membrane by carnitine/acetylcarnitine translocases (Vaz and Wanders 2002).

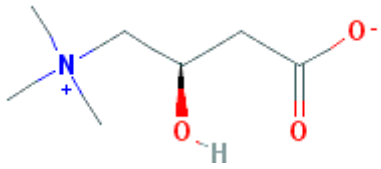

Figure 4.1: The chemical structure of L-carnitine. Image obtained from Pubchem (http://www.pubchem.ncbi.nlm.nih.gov).

As a result of its involvement in the transfer of fatty acyl chains, carnitine has a wide spanning influence on various cellular processes and on energy homeostasis in general (Calabrese *et al.* 2006). Specifically, carnitine has been found to be involved in the maintenance of cellular acyl-CoA pools as well as in the depletion of toxic fatty acyl-CoA residues (Malaguarnera 2012). Due to its free hydroxyl group, it has been suggested that carnitine could act as a molecular chaperone (Pettegrew *et al.* 2000) and also that it may interact with membranes, causing changes in their physical properties (Virmani and Binienda 2004).

In addition, there have been various studies indicating that carnitine supplementation results in a protective effect against oxidative stress (Li *et al*. 2012; Yildirim *et al*. 2013; Silva-adaya *et al.* 2008). These effects have since been of particular interest due to the presence of oxidative stress in many diseases, including hypoxia, ischemia and neurodegenerative disease (Pettegrew *et al*. 2000). Due to these findings, carnitine supplementation has also been studied as a potential treatment for numerous of these pathologies (Schöls *et al.* 2004; Sorbi *et al.* 2000; Thal *et al*. 2000).

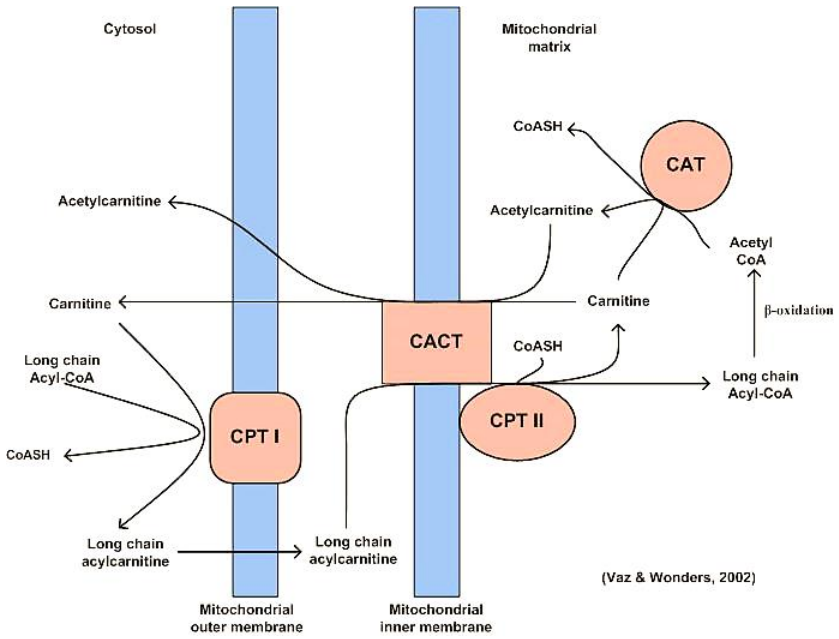

Figure 4.2: The carnitine shuttle is responsible for the transport of fatty acyl residues through the action of carnitine palmitoyltransferase (CPTI and II), carnitine acetyltransferase (CAT) and the carnitine/acetylcarnitine translocase (CACT). (Adapted from Vaz & Wonders, 2002).

Recent studies in *Saccharomyces cerevisiae* indicate that the protective role of carnitine against oxidative stress occurs independently of its role in the carnitine shuttle (Franken *et al.* 2008; Franken and Bauer 2010). Various potential mechanisms for the observed action of carnitine have been suggested, including that of carnitine interacting with membranes, increasing stability (Virmani and Binienda 2004), that acetyl-carnitine may aid in neurotransmission due to its structural similarity to acetyl-choline (Nałecz *et al.* 2004), through modulation of proteins by transacetylation (Pettegrew *et al.* 2000) or alteration of gene expression of cytoprotective genes (Traina *et al.* 2009). However, the cellular and molecular mechanisms underlying the protective effect of carnitine remain largely unknown.

Recent studies on yeast in this laboratory have shown not only a protective effect of carnitine against oxidative stress, but also a detrimental effect of carnitine supplementation when in combination with the thiol reducing agent dithiothreitol (DTT) as well as the thiol oxidising agent diamide (Franken and Bauer 2010). However, although some protective effects of carnitine against oxidative stress in mammalian cells have been described (Li *et al.* 2012; Silva-adaya *et al*. 2008), the exact mechanisms involved and the effect of treatment together with DTT, are at this time still unknown. In order to determine whether carnitine supplementation produced similar phenotypes as observed in yeast, which could be indicative of a common mechanism of action, the aims of this study were to investigate the effects of carnitine on toxicity induced by hydrogen peroxide, DTT and paraquat in a mammalian system. Paraquat is a bipyridyl herbicide which is thought to potentiate loss in viability through induction of ROS production (García-Rubio *et al*. 1998).

The yeast *S. cerevisiae* has been proposed as a model for the study of neurodegenerative disorders (Tenreiro and Outeiro 2010). However, there are limitations to the use of yeast for this purpose and comparative studies are therefore vital to determine the extent to which this model could be used. Considering the phenotypes observed in *S. cerevisiae*, this study made use of neuronal cells to assess whether similar phenotypes could be observed in a mammalian system. Neural cells were chosen for this purpose due to the high degree of oxidative phosphorylation that occurs within these cells and also because of the link between the effects of oxidative stress and several neurodegenerative disorders.

4.2 Methods and Materials

4.2.1 Cell culture

For the purposes of this study, the GT1-7 mouse neuronal cell line of hypothalamic origin was used. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. Growth medium was changed every 48 hours. Cells were incubated at 37 °C and 0.5% carbon dioxide.

4.2.2 Viability assays in mammalian cells

Cultures were grown to 70-80% confluency and then transferred to 48 well plates at 9 x 10^5 cells per well. The cells were then grown for 24 hours prior to treatment with; 1mM DTT, 200 µM hydrogen peroxide or paraquat (0.5 mM and 1 mM) in the presence or absence of supplemented carnitine and incubated for six hours. Pre-incubation with carnitine was also tested where cells in the carnitine treated group were first incubated for 24 hours in the presence of carnitine prior to the addition of either DTT (Sigma) or paraquat (Sigma) together with carnitine (Sigma) as described above. The medium was then removed and 200 µl MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) in PBS (phosphate buffered saline) was added. The cells were then incubated for two hours. The MTT solution was removed and 200 µl isopropanol-Titon X solution was added. Absorbance was measured using a microplate reader (EL-800, Biotek Instruments) at 595 nm.

4.2.3 Extraction of carnitine from cells

The extraction of carnitine was carried out as previously described (Canelas *et al*. 2009) with some adaptations (Franken *et al.* 2015). Briefly, cells were grown until 80% confluency and trypsinized. Approximately 6 x 10^6 cells were used for the extraction. These were spun down and washed once in PBS. At this point cells were weighed for the calculation of wet weight. Cells were then resuspended in boiling buffered ethanol (75% ethanol, 10 mM HEPES, pH 7.) and incubated at approximately 95 °C for three minutes. Thereafter, samples were spun down and the supernatant was transferred to a new tube. Samples were dried under vacuum and then resuspended in 15 %

methanol. Prior to analysis, 100 µl C^{13} labelled carnitine (final concentration of 10 ppm) was added to 400 µl sample and 500 µl acetonitrile.

4.2.4 HILIC-ESI-MS

Analysis of the carnitine content of cells and media was performed on a Waters Acquity UPLC (Waters, Milford, MA, USA), connected to a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer fitted with an electrospray ionisation probe. Carnitine was detected in the positive mode using a capillary voltage of 2.5kV, cone voltage of 15V, desolvation gas (nitrogen) flow rate of 650L/hr. at 275°C. Separation was carried out on a Waters BEH Amide 2.1x100mm, 1.7um particle size column, at 40°C, a flow rate of 0.5mL/min was applied and 2µL were injected. Solvent A (20mM NH_4OAc, 20 mM NH_4OH) was made up in water and solvent B (20mM NH_4OAc, 20 mM NH_4OH) in 95% acetonitrile. The gradient started at 10% solvent A for the first 0.5 min, changing to 15% A in the next 1.5 min and then 60% A over the next 3.5 min. The column was re-equilibrated for 4.5 minutes, leading to a total run time of 10 minutes. Data was analysed using Masslynx version 4.1.

4.3 Results

4.3.1 Viability assays

Exposure of cells to carnitine had no significant effect on cell viability for the time period and concentrations tested (Figure 4.3). Addition of DTT resulted in a significant decrease in cell viability compared to the control [32.5 ± 5.6% ($p< 0.05$)]. This response was neither rescued nor augmented to a significant degree by the addition of carnitine (Figure 4.4). The addition of paraquat to the growth medium also resulted in a significant decrease in cell viability compared to the control [41 ± 5.5% ($p< 0.05$)]. Carnitine supplementation together with paraquat, as with DTT, did not result in any significant changes in cell viability as observed in the MTT assay (Figure 4.5). Experiments were also performed using hydrogen peroxide as oxidative stress inducing agent. However, due to the high variability in results obtained when using this compound, no further analysis was carried out (appendix A).

In order to allow sufficient time for the cells to respond to the presence of carnitine before addition of DTT or paraquat, it was also assessed whether pre-supplementation of carnitine 24 hours prior to the addition of paraquat or DTT would lead to a protective or detrimental response, respectively, as seen in yeast. However, although a significant reduction in cell viability for paraquat-treated cells was observed, [78 ± 5.2% ($p< 0.05$)] no significant effect was seen for the co-supplementation of carnitine with paraquat (Figure 4.6). Results for pre-incubation with carnitine on DTT toxicity were inconclusive as no effect for the DTT treatment alone under these conditions was observed (Appendix A).

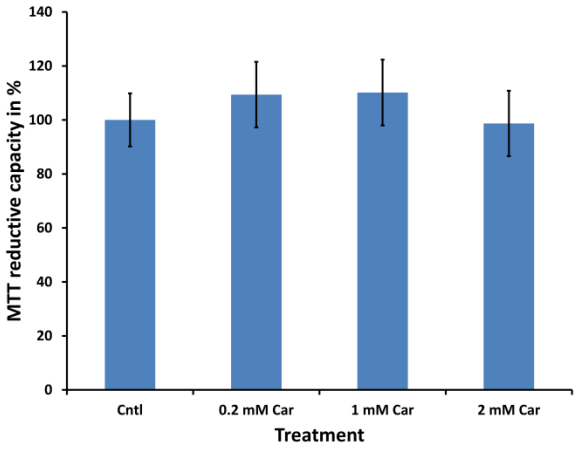

Figure 4.3: No significant changes in cell viability were observed in response to carnitine supplementation (Car) at various concentrations after incubation for six hours when compared to the control (Cntl). Data shown as mean ± SEM of two independent experiments (n = 2).

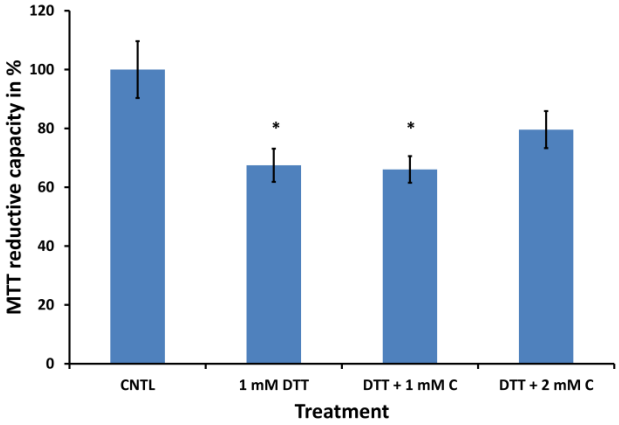

Figure 4.4: Treatment with 1 mM DTT significantly reduces reductive capacity after six hours, an effect that is not significantly altered in either a protective or detrimental manner by the presence of supplemented carnitine (C) in comparison with the control (CNTL). A slight increase in reductive capacity was observed when 2 mM carnitine was co-supplemented with DTT. However this was not a significant change. Data shown as mean ± SEM of three independent experiments (n = 3) (*p < 0.05 vs. control).

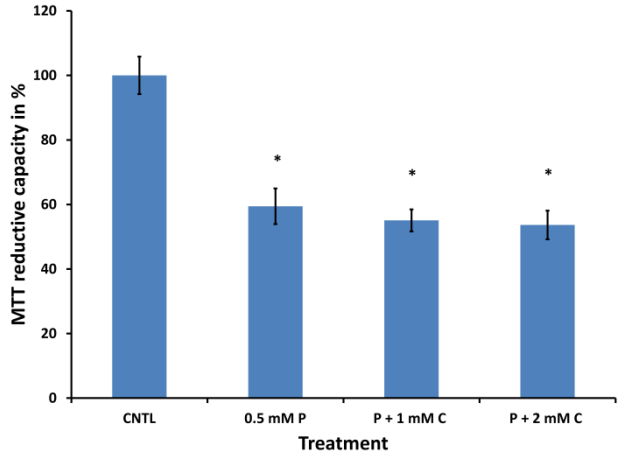

Figure 4.5: The addition of 0.5 mM paraquat (P) resulted in a significant decrease in viability after six hours incubation which was not significantly impacted by the co-supplementation of carnitine (C) compared with the control (CNTL). Data shown as mean ± SEM of three independent experiments (n = 3) (*p < 0.05 vs. control).

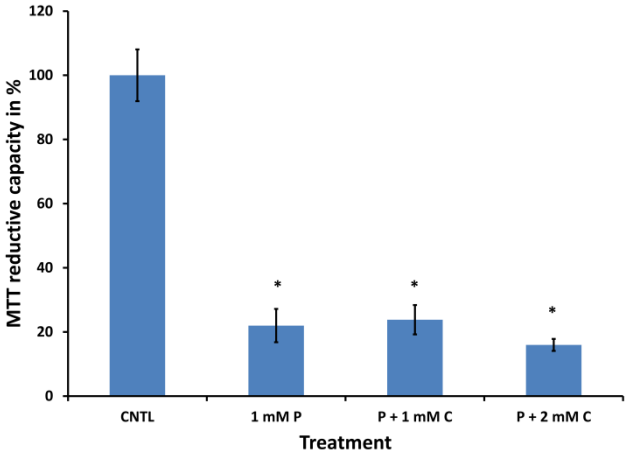

Figure 4.6: Incubation with paraquat (P) for six hours led to a significant decrease in reductive capacity and was unaffected by the presence or absence of carnitine (C) exposure for 24 hours prior to the treatment with both carnitine and paraquat for 6 hours. Data shown as mean ± SEM of three independent experiments (n = 3) (*p < 0.05 vs. control).

4.3.2 Determination of carnitine concentration in cells and media

Although it is known that L-carnitine is mainly produced in the liver and kidney of mammals (Hoppel and Davis 1986), the enzymes involved in carnitine biosynthesis have also been found in the brain (Vaz and Wanders 2002). However, not much data is available regarding the ability and extent to which individual cell lines are able to synthesize this compound. An additional source of carnitine is the foetal calf serum which is supplied to cells in the growth medium. LCMS was thus performed in order to determine the amount of carnitine present in the cells and growth medium. The carnitine concentration extracted form cells was 1.1 ± 0.05 µM. In contrast, the concentration of carnitine in the medium was found to be 2.8 µM.

4.4 Discussion

Carnitine has been found to be protective against oxidative stress both in yeast and mammalian cells (Franken and Bauer 2010; Silva-adaya *et al*. 2008). However, the mechanism underlying this action and whether it is shared between yeast and mammals remains unknown. Supplementation of carnitine on its own was found to have no effect on cell viability, a finding which is in agreement with previous work (Li *et al*. 2012). However, a detrimental effect of carnitine supplementation has also been previously reported where carnitine was found to increase vulnerability of the brain to oxidative stress, although only at higher concentrations (e Luz *et al.* 2013)

The decrease in cell viability due to the addition of paraquat was as expected and has been previously reported (García-Rubio *et al*. 1998). Due to the numerous reports showing a protective effect of carnitine supplementation against oxidative stress in previous studies (Li *et al*. 2012; Yildirim *et al.* 2013; Silva-adaya *et al*. 2008), it was surprising to find that carnitine had no effect on cell viability when supplemented either together, or 24 hours prior to addition of paraquat to the

growth medium. Conversely, one study has previously reported that carnitine, supplemented 30 minutes prior to paraquat, increased the negative impact on cell viability after three hours (García-Rubio *et al.* 1998). However, the concentration of carnitine used in that study (50 mM) was very high compared to what was used in this study (1 and 2 mM) and in other work (Li *et al*. 2012) and therefore it is possible that these results may have been influenced by other factors such as osmotic stress and not solely to a specific action of carnitine itself.

Furthermore, unlike in the current study where neuronal cells were used, the above mentioned work was done in hepatocytes and therefore some differences in results may be attributed to cell type-specific responses to oxidative stress and the supplementation of carntine as has previously been reported (Sachan *et al*. 2012). A detrimental effect of carnitine has been reported in yeast in combination with the thiol modifying agents DTT and diamide (Franken and Bauer 2010). However, although paraquat has also been seen to lead to a loss in cell viability in yeast, the combination of paraquat and carnitine has not thus far been investigated and it is thus uncertain how the responses for the two systems would compare in this instance.

Treatment of cells with DTT for six hours at 1 mM led to an expected decrease in viability. Previous studies have found DTT to be toxic between 0.4 and 1 mM but not at higher or lower concentrations. This is indicative of very specific and controlled parameters in the toxicity of this agent for mammalian cells and may also explain why results for experiments with 24 hour pre-treatment of carnitine led to inconclusive results. No significant effects were seen when carnitine was co-supplemented with DTT, however co-supplementation of 2 mM carnitine together with DTT resulted in a slightly increased reductive capacity, indicating that higher concentrations of carnitine may be capable of protecting against toxicity induced by DTT. As no studies on the co-supplementation of DTT and carnitine in the mammalian system have thus far been carried out, these results could not be compared to previous work.

The lack of a significant response when cells treated with both DTT and paraquat are supplemented with carnitine could be due to the intrinsic levels of carnitine being sufficient for any role this molecule might play under these conditions. However, both the concentration of carnitine in the cells and that of the medium were far lower than the concentrations utilized in this study as well as in previous studies investigating the impact of carnitine supplementation together with oxidative stress inducing agents (Li *et al*. 2012). It was thus determined that the concentration of carnitine present prior to supplementation was not such as would render the addition of carnitine at concentrations used in this study insignificant.

However, although the concentration of carnitine determined within the cells and medium was far below the concentrations supplemented in this study, the fact that the carnitine within the cells was lower than that present in the medium implies that levels of carnitine already present were sufficient, at least for the normal functioning of the cells, and this would mask any effects carnitine

may induce when in combination with paraquat and DTT. Some previous studies have made use of medium lacking foetal calf serum when treating cells (Li *et al*. 2012). This condition, which is known as serum starvation could perhaps decrease the availability of carnitine in the medium and may make the carnitine-associated phenotypes more apparent.

In conclusion, although no effects of carnitine supplementation either positive or negative were found in this study, the wealth of data from previous studies showing the involvement of carnitine in protection against oxidative stress warrants further investigation. Future studies could investigate the possible use of medium lacking carnitine, or the removal of serum prior to treatment. Also, as the results indicate a trend of increased viability in cells treated with DTT and 2 mM carnitine, the effects of a further increase in carnitine concentration should be investigated. The effect of carnitine on treatment with paraquat has not yet been tested in yeast and should be determined in future studies.

In addition, it would be beneficial to explore other methods of analysing carnitine phenotypes which are more relatable to those used in the yeast studies for the production of more easily comparable results. In yeast, growth rate, as opposed to survival is most often studied. Therefore, use of approaches such as flow cytometry to analyse the effects of carnitine on cell proliferation under redox stress may reveal responses which could not be observed by the use of the methods employed in this study.

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APPENDIX A

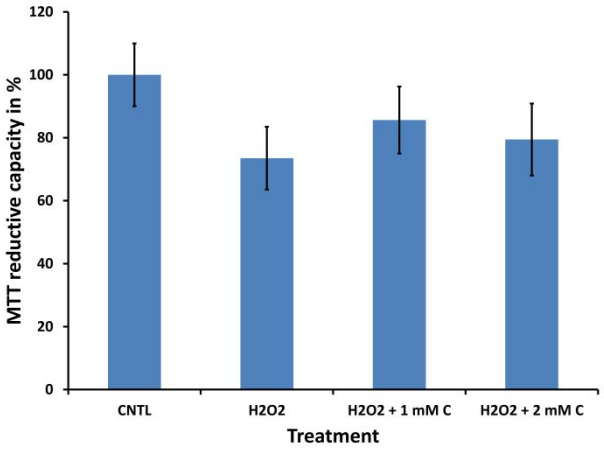

Figure A1: Exposure of cells to hydrogen peroxide for six hours alone and in combination with carnitine (C) did not lead to a significant reduction in cell viability compared with the control (CNTL). Data shown as mean ± SEM (n = 1).

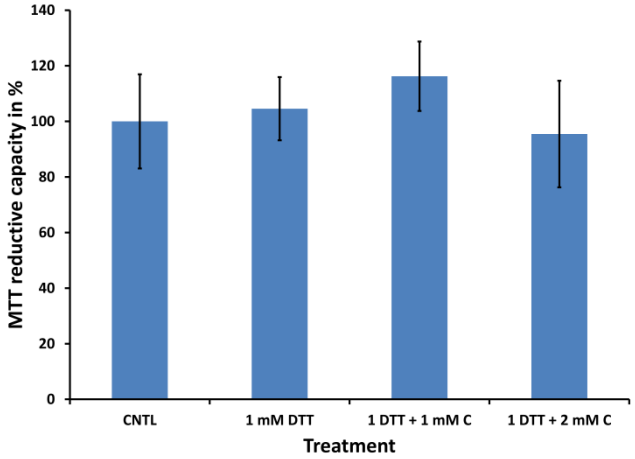

Figure A2: No significant differences in cell viability were observed in cells treated with DTT (D) alone and in combination with carnitine (C) on cell viability after exposure to carnitine for 24 hours prior to exposure to both carnitine and DTT for 6 hours compared with the control (CNTL). Data shown as mean ± SEM for two independent experiments (n = 2).

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

5.1 General discussion and conclusions

Although carnitine has been widely studied for its protective role against oxidative stress, the mechanism by which this takes place still remains largely unknown. Various actions for carnitine have been suggested including modulation of gene expression, modification of proteins and alteration of membranes (Arienti *et al*. 1992; Madiraju *et al*. 2009; Traina *et al*. 2009). However, none of these could conclusively account for the effects of carnitine against oxidative stress. Studies in this lab have found that Δ*cho2* and Δ*opi3* are less responsive to carnitine supplementation and, therefore, the role of choline in the carnitine phenotypes was investigated.

The effects for choline supplementation to the wild type did not result in any significant phenotypes; however, addition of this compound to the PC synthesis mutants not only increases growth but also restores the effects of carnitine when combined hydrogen peroxide or DTT treatment. These results indicate that choline is involved, or required for shuttle-independent carnitine-dependent phenotypes without inducing a similar effect to that of carnitine supplementation. This latter hypothesis was indeed a possibility when considering the structural chemical similarity between the two compounds. Choline is also known to be involved in neurotransmission in mammals, but in yeast this compound has thus far been linked only to lipid metabolism as a precursor of phosphatidylcholine (Henry *et al*. 2012).

Little is known about the mechanisms cells employ in phospholipid distribution and homeostasis at present (De Kroon *et al.* 2013). However, the effects of changes in cell membrane phospholipid composition on resistance to environmental stress factors has lately become of great interest and could provide valuable information which is applicable also in the study of various human diseases (Santos and Riezman 2012).

The analysis of cellular lipid extracts indicated that the mutants displayed an expected decrease in phosphatidylcholine levels accompanied by increased phosphatidylinositol and TAG. However, no major change in the phospholipid composition of wild type cultures treated with carnitine, choline, DTT or hydrogen peroxide was observed. These results suggest that the phenotypes observed in the liquid and plate assays are not due to an ability of carnitine to directly influence the cellular phospholipid composition.

Due to the association of neurodegenerative disorders with oxidative stress, the protective role of carnitine under these conditions has also been of great interest in this field of study (Montiel *et al*. 2006; Silva-adaya *et al*. 2008). However, no significant effect of carnitine supplementation was observed in the mammalian cell cultures when treated with either paraquat or DTT in this study. These results, which conflict with previous work (García-Rubio *et al*. 1998; Li *et al*. 2012) could be due to cell-type specific responses to carnitine supplementation. In addition, the presence of

carnitine in the medium and within cells could be concealing any effects which carnitine might have had.

5.2 Future work

Future work should focus on further investigation into the growth-slowing effect of carnitine and its possible link to the hydrogen peroxide and DTT phenotypes. Also, further study into the effects of deletion of *OPI3* and *CHO2* on the cell as well as how this is affected by supplementation with choline would be of value. The analysis of the lipid composition of cells in this study did not show any effect for carnitine supplementation, however not all aspects were covered and future work should also include analysis of the stoichiometric relationships between the phospholipid composition as well as investigating the effects of oxidative stress and carnitine supplementation on the phospholipid distribution in membranes. Lastly, the optimizing of analysis in mammalian cells such as flow cytometry for more easily comparable results between yeast and mammalian systems would be beneficial. Also, reducing other sources of carnitine to the cells could aid in clarifying the effects of carnitine against oxidative stress would be worthwhile to investigate.

5.3 References

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